US009228193B2

(12) **United States Patent**
Surzycki et al.

(10) **Patent No.: US 9,228,193 B2**
(45) **Date of Patent: Jan. 5, 2016**

(54) **SYSTEM, METHOD, AND DEVICE FOR THE EXPRESSION OR REPRESSION OF PROTEINS**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 1032 days.

(21) Appl. No.: **12/377,238**

(22) PCT Filed: **Aug. 11, 2007**

(86) PCT No.: **PCT/US2007/017774**

§ 371 (c)(1),
(2), (4) Date: **Feb. 11, 2009**

(87) PCT Pub. No.: **WO2008/021223**

PCT Pub. Date: **Feb. 21, 2008**

(65) **Prior Publication Data**

US 2010/0184180 A1 Jul. 22, 2010

Related U.S. Application Data

(60) Provisional application No. 60/837,001, filed on Aug. 11, 2006.

(51) **Int. Cl.**
C12N 15/82 (2006.01)

(52) **U.S. Cl.**
CPC ***C12N 15/8214*** (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

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(74) *Attorney, Agent, or Firm* — Barnes & Thornburg LLP

(57) **ABSTRACT**

This invention relates to systems, methods, and devices for inducing and/or repressing the expression of proteins. More particularly, the invention relates to systems, methods, and devices for inducing and/or repressing the expression of proteins in plastids. An exemplary embodiment involves the regulation of the expression of proteins involved in hydrogen production to stimulate the production of hydrogen gas using the methods, systems, and devices described herein.

14 Claims, 38 Drawing Sheets

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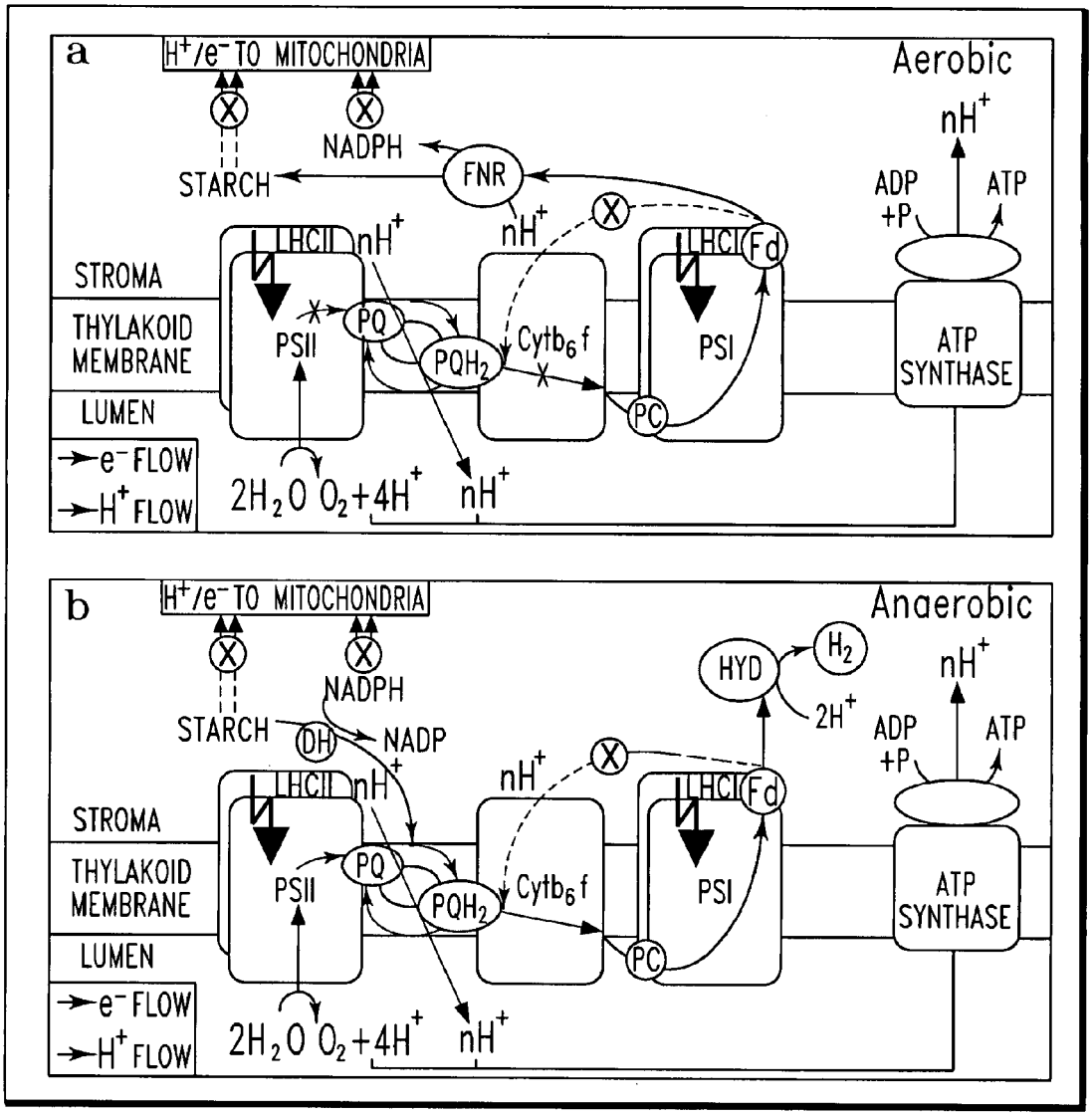
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Aerobic
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FNR
nH+
ADP +P
ATP
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LHCI
Fd
STROMA
THYLAKOID MEMBRANE
LUMEN
PSII
PQ
PQH2
Cytb6 f
PSI
PC
ATP SYNTHASE
e- FLOW
H+ FLOW
2H2O O2+4H+
b
H+/e- TO MITOCHONDRIA
Anaerobic
NADPH
STARCH
DH
NADP+
HYD
H2
2H+
nH+
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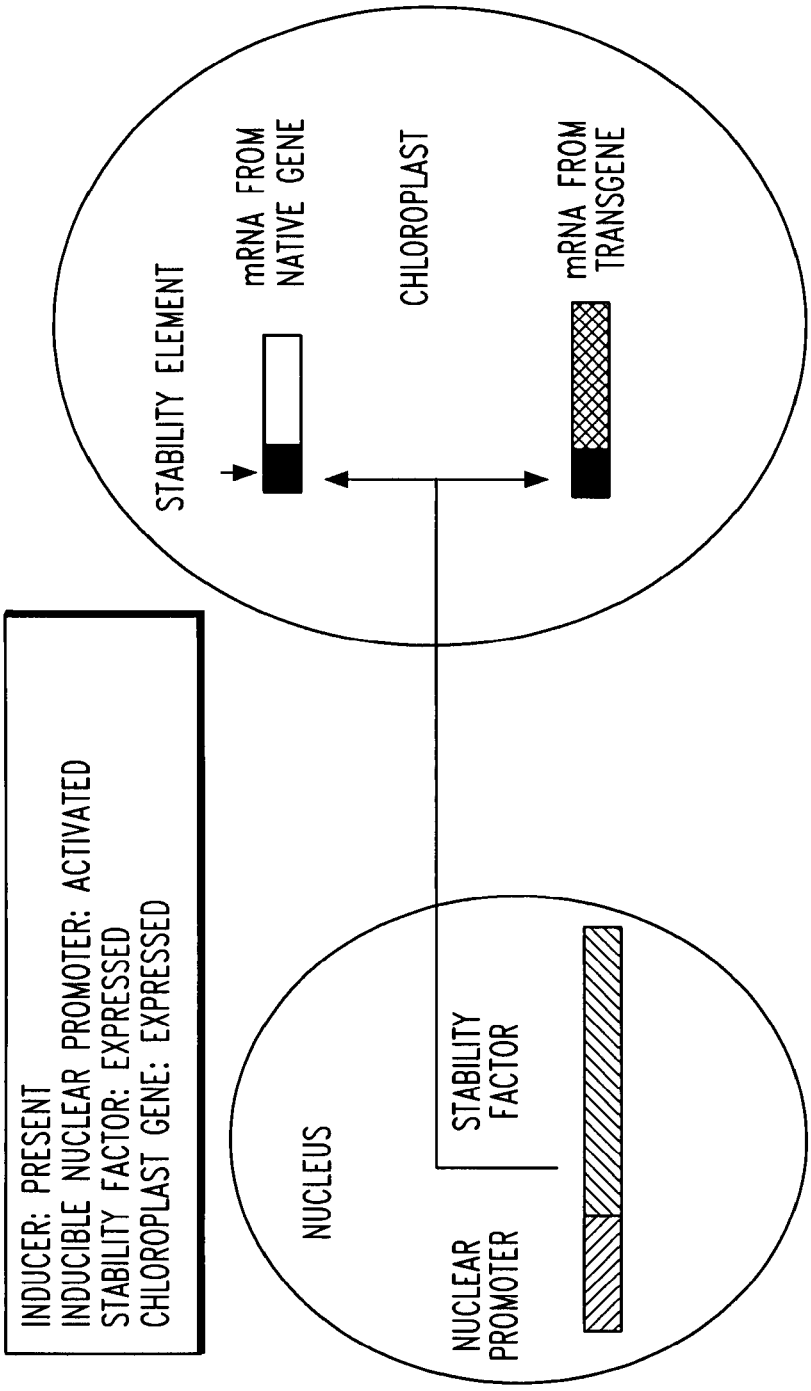
Fig. 1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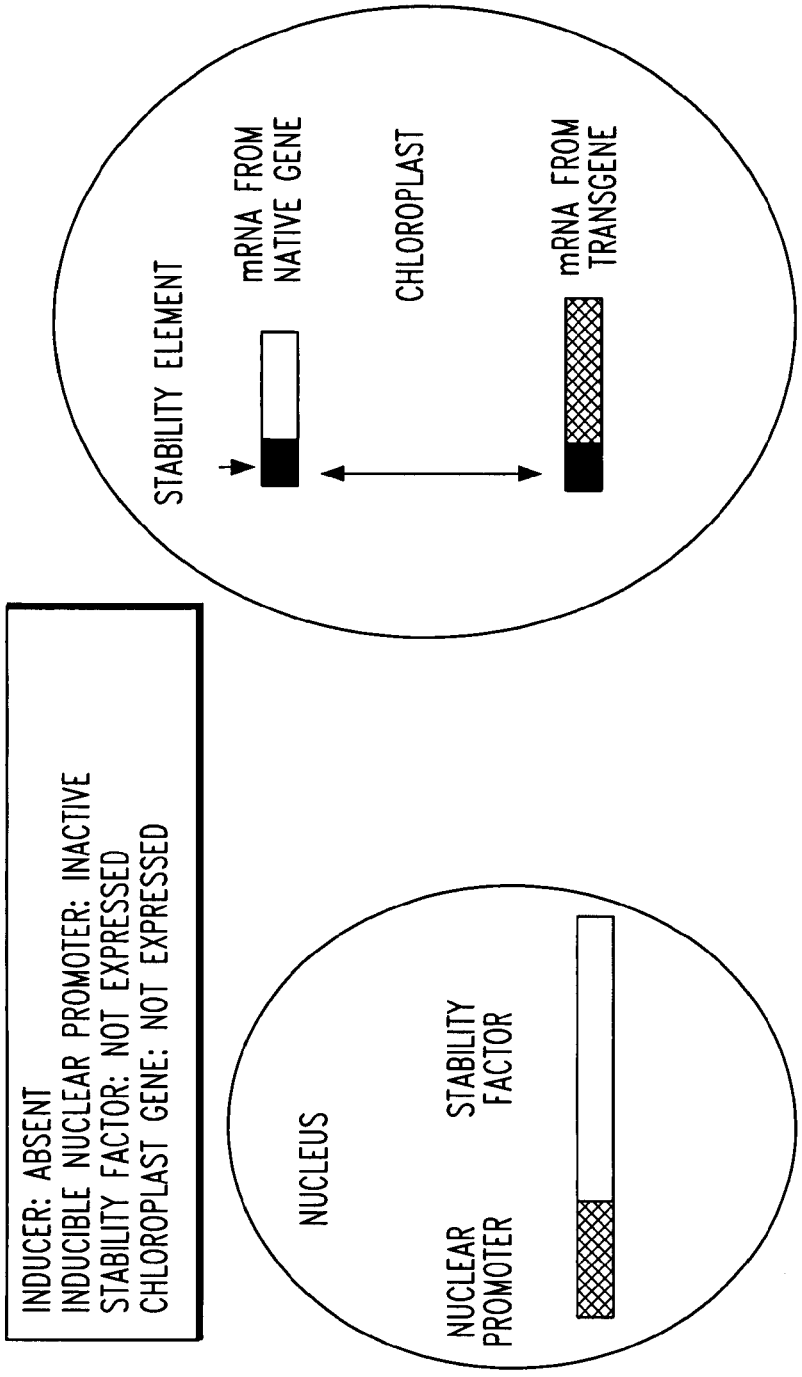
CHLOROPLAST
STABILITY ELEMENT
mRNA FROM NATIVE GENE
mRNA FROM TRANSGENE

Fig. 2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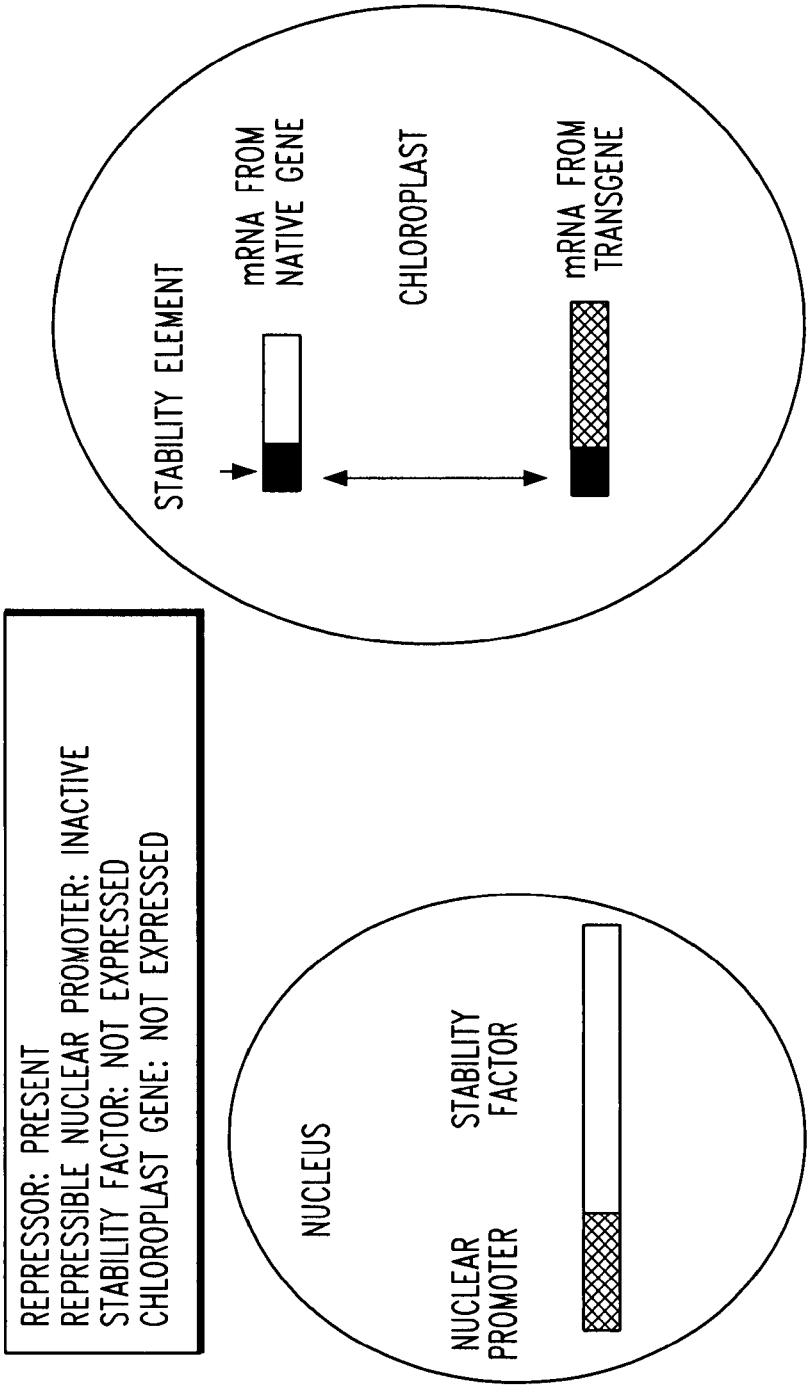
CHLOROPLAST
mRNA FROM TRANSGENE

Fig. 3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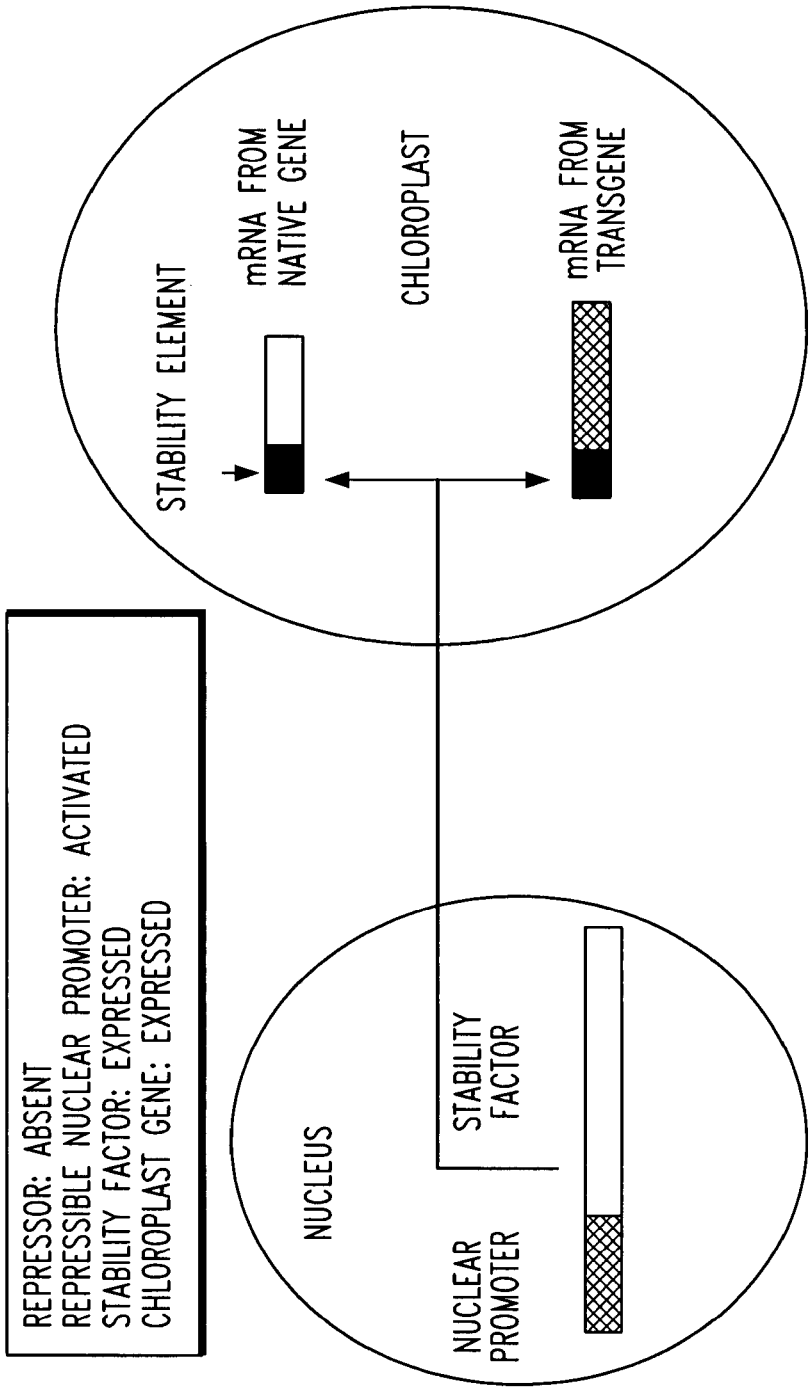
STABILITY FACTOR
STABILITY ELEMENT
mRNA FROM NATIVE GENE
CHLOROPLAST
mRNA FROM TRANSGENE

Fig. 4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CHLOROPLAST
STABILITY ELEMENT
mRNA FROM NATIVE GENE
mRNA FROM TRANSGENE

Fig. 5

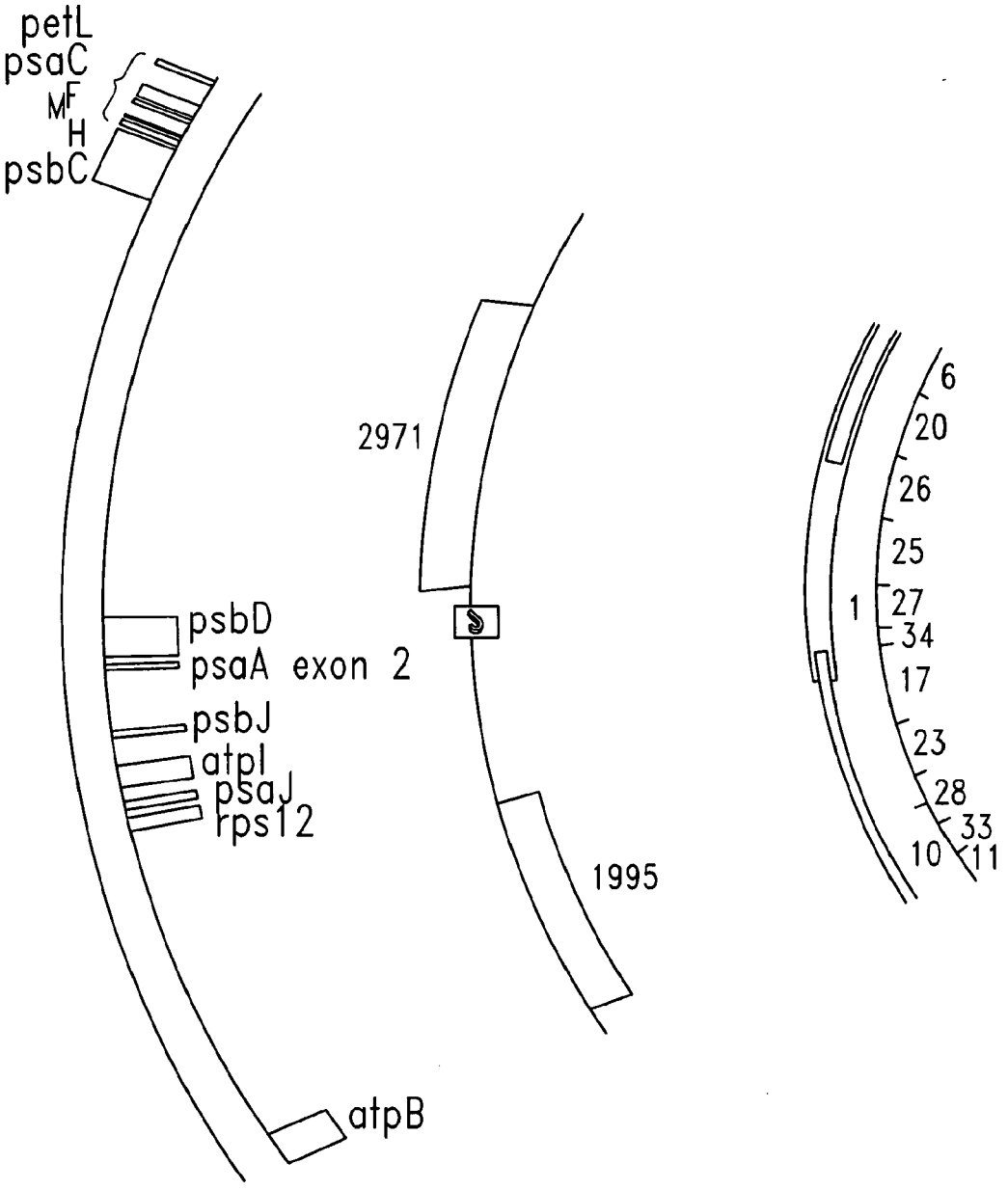
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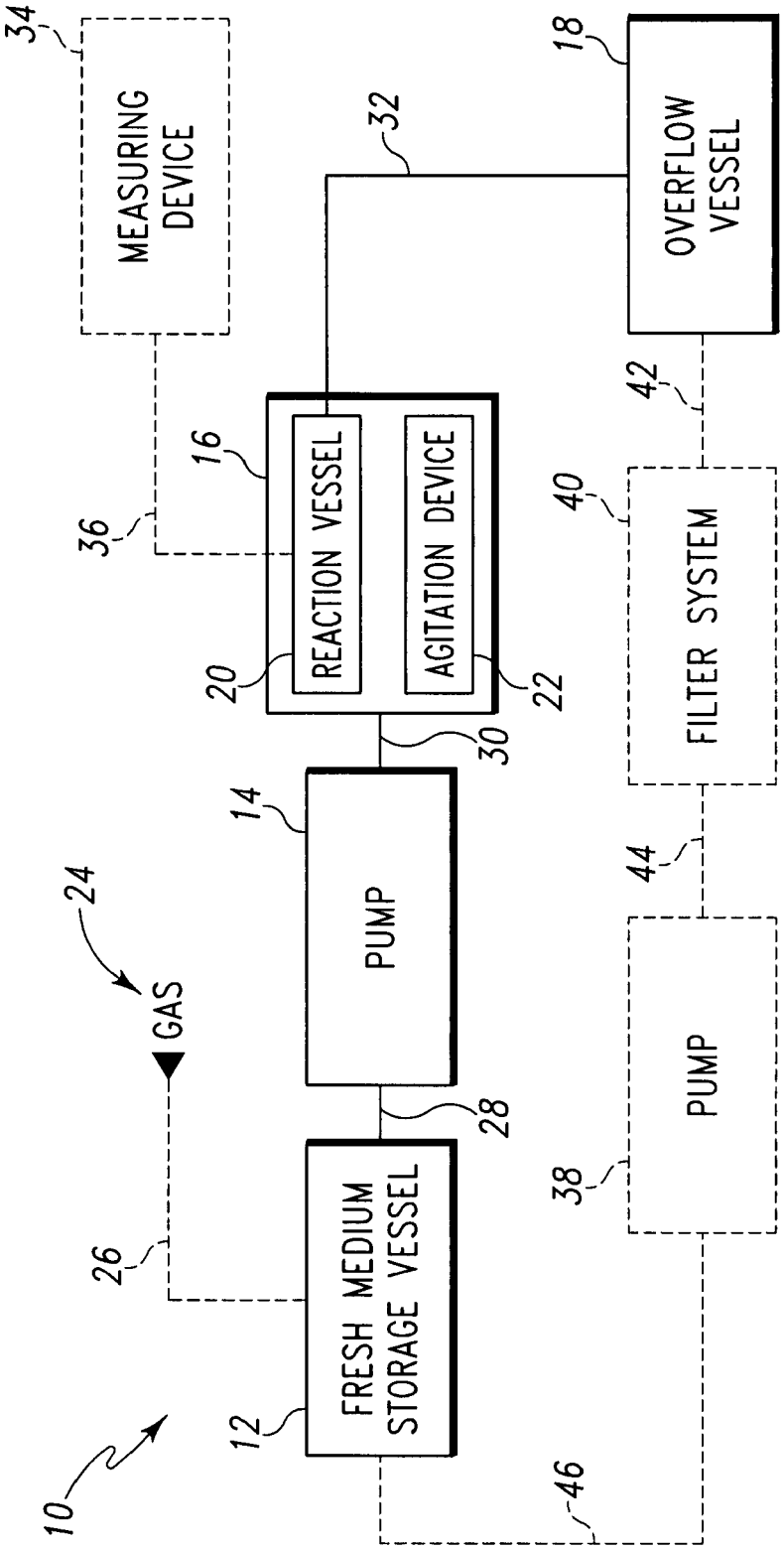
Fig. 6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AGITATION DEVICE
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PUMP
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GAS
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FRESH MEDIUM STORAGE VESSEL
12
10
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Fig. 7

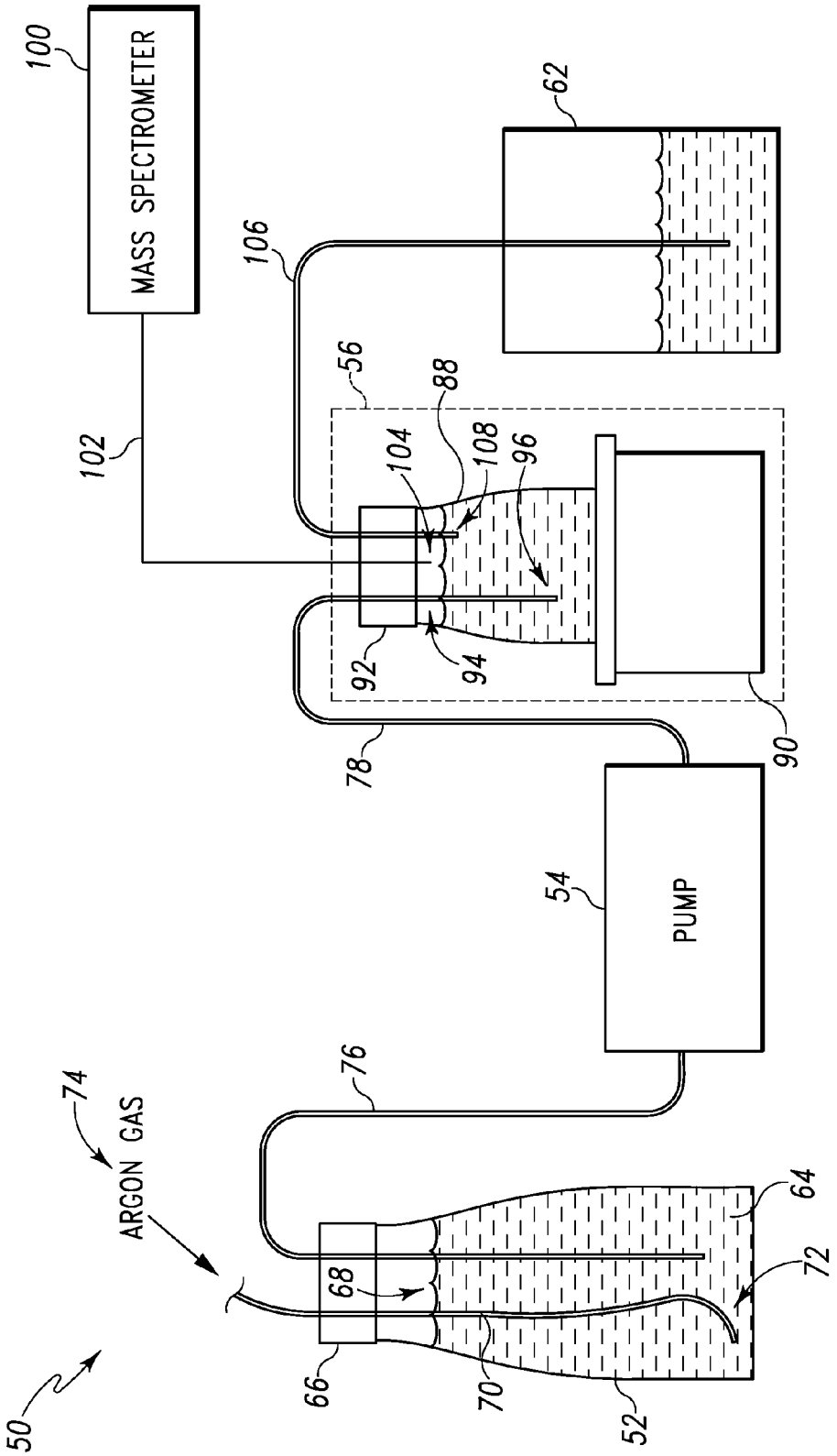
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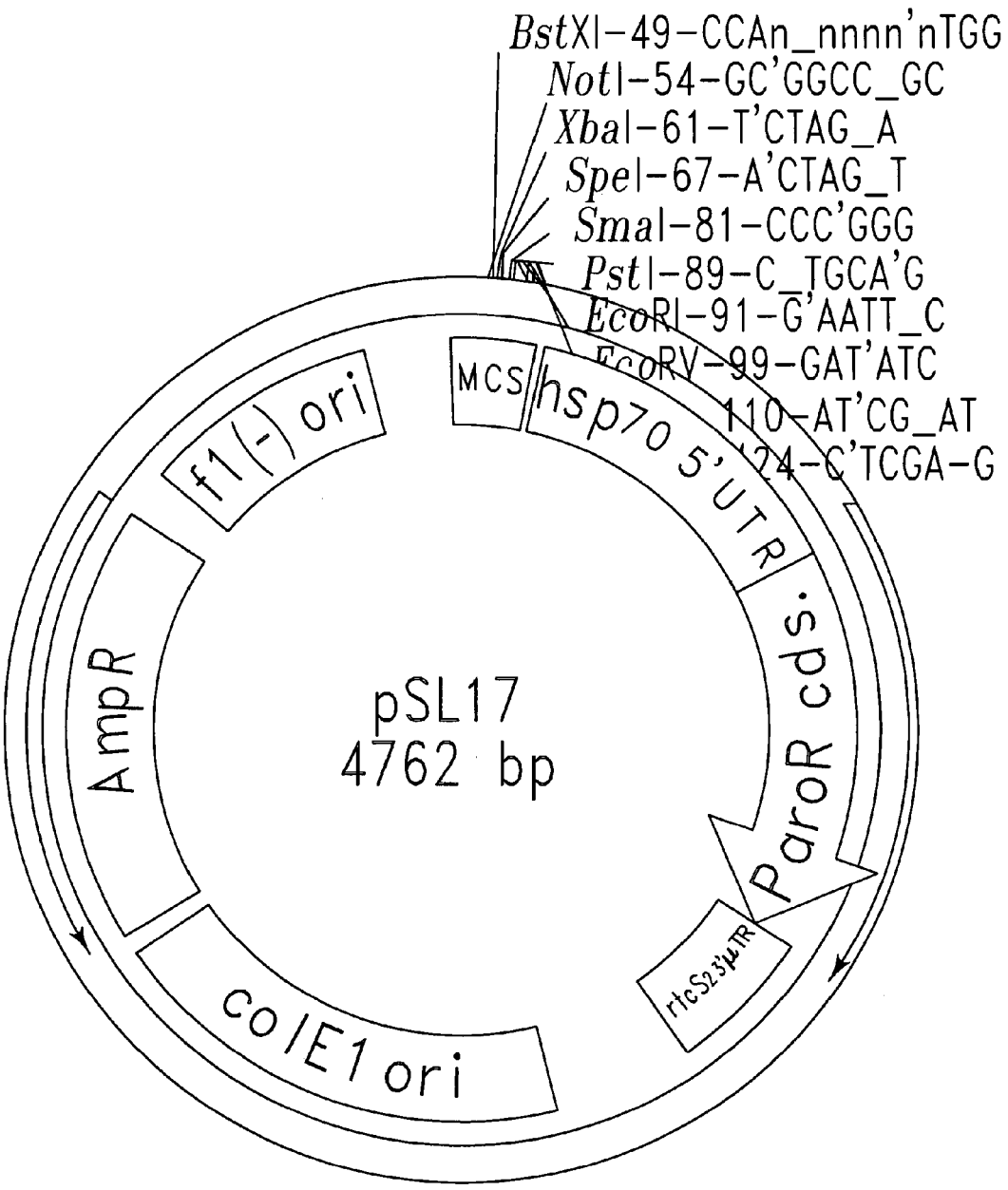
Fig. 8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PstI-89-C_TGCA'G
EcoRI-91-G'AATT_C
EcoRV-99-GAT'ATC
110-AT'CG_AT
124-C'TCGA-G
MCS
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f1(-) ori
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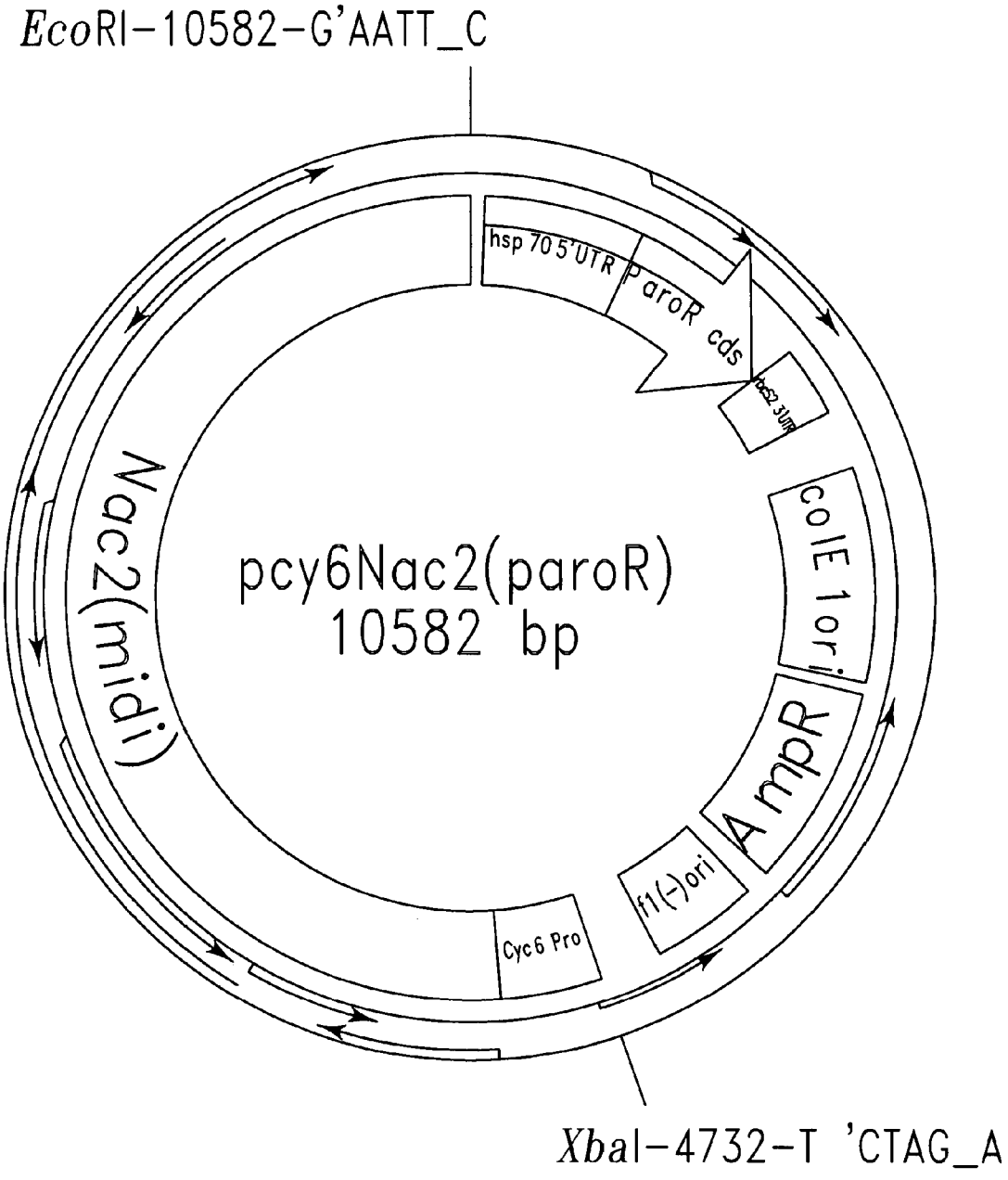
4762 bp
ParoR cds.
rtcS23'μTR
colE1 ori

Fig. 9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AmpR
f1(-)ori
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Nac2(midi)
pcy6Nac2(paroR)
10582 bp
XbaI-4732-T 'CTAG_A

Fig. 10

1	GAATTCTGCT	GCGGAAAGGT	TAACTGGCCC	AAAGACTTGC	GGCGCAGCGC
51	TGAAATAGCT	CCCAAAACCT	AGATACGCTC	CGTCATGAAA	CAAAACATGT
101	ATATATTAGC	AAAAGGGCGT	GACTGCCGGG	GCGAGCGGCC	TCGCCCAGCC
151	GATCTCTCCA	TCGTGCTTGC	TGTCACCGCT	CGCTGCTCAA	CACAGGCTAA
201	CTTAGCTTGC	CTTTGGTAGA	CATTGTGCAA	TCAGTCACAC	CTCAAGCGCT
251	AATCTTCAAA	ATCTTTCGGT	CGACTGAAAC	GATAGTGAGC	GTCTCAGAAC
301	GAGTCTTGTG	TAGGCCGCAC	GTTTGAAGCG	GTCGGGGCCG	TGTCCAACGT
351	ATAGGTTGCT	GTTATCAAGA	AGAAACAAAC	CAGCTGGTAG	CAAACCTCGA
401	ACGAAGTATG	GCACGGCCAT	AGAAGCCCGG	CCTTGCGGGA	GAACTCCGGA
451	AA*ATGGGGGC*	*TCTACCGTGC*	*CCAGCTCATA*	*TCGAGCATCA*	*TCAGGGCTTA*
501	*TCATCGTTTG*	*GGACACGGCG*	*AGTCCTGCGG*	*CAAAGCGTGG*	*CTTGTGGAGC*
551	*TCACCGCAGT*	*CGGCGAAGGT*	*CCCTGTGGGC*	*AGGCGCT*TCT	CCCGGCGCAC
601	CTAAATATGA	CGAGGGCGCA	TTATCGTGTA	GCTTTCAACT	GGGCTCGGCG
651	AGCCTGCACG	TCAGCGAGGT	AGGCTTGTTT	TCTTGCCACA	GTGTATGGGT
701	GGCTCGGGAG	ACCTGGGAGA	TCGGAGCGTT	GGGTGTTCGG	GGCTCGCGAA
751	TGGTGCAGAA	ACAAGCTTCG	TGGCATTTAA	GGCGTCCCGC	GACAATAAAC
801	GCAGGTTCGA	GGTTCGCGAC	ACGCGACACC	AAGGGCCCCT	CCAACCGGCG
851	GATGTATCGA	GCTCCCGAAA	ACTCTCGCGC	TCACGGAGGA	GCAGGACCAG
901	CGGGCTGCGC	GGGCGACCAG	CCAGCTGCTA	AATGCCATCA	GTCGCTGTGC
951	CGCGGGCTCG	CCTTTGGCGC	GCTTACTGAC	TGGTCCTCAT	GGAGCCGCGA
1001	GCGCAACTGG	TGCCGGGAGC	CACTCGTCGT	CGGCCGGGGC	GCCCACGCCG
1051	ACGCCGCGGC	CGGACATCGC	GGCCGCTGCA	GCGCTGCTCC	CCTCGCTGAC
1101	TTGGCGCTGG	GTGTCGGTGG	CGGACCTGGA	AGGAGGCTGG	GTTGACCGCG
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1201	GACGTCAACG	CATCCGTCAG	GTGGGCAGCA	CCGCGTTCTT	AAGGCGTGAC
1251	CTGCCGTACA	TTGCTGGCAG	GGCTCTGTGG	TATGGAGCCA	TGGATGTCCG
1301	TTGCAGTTTG	TTGACTCAGC	ACGCGCTGGC	AGGCTGACAC	GCTGCGGCAT
1351	GTGTGCACGT	CCGGAAACGT	TCCTTGCGCG	AGCTGGGCTC	AACCTGACAT
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1451	GAGCTGGTCG	GGTGGTGCAT	GGACAACTGC	AGCGCACCCG	CGGCGGCACT
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1551	GCAGCTTCTA	CGGCCTGCTG	CTTCAGCGCC	TGGACGGCGG	TGGCGCCAGC
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2201	CCTGCCGCGC	TTCTCCTGAA	GCGCCCGTCC	CGCAGCGCCG	CCCCGCCAGC
2251	ACCAACGCTG	CCCGACGGTG	CGGAGGCGGA	CGGATTCGCA	TCGGACGGGA
2301	GCGGCCTGCC	AGGACACGGG	CAGCAGCAGC	TCTCCGCCAT	TGCGGCGGCG
2351	GCAGCAGCCT	CATCGCTCCT	GGCCGGCCCG	GCGGCGCCCA	TGTCGTTCCT
2401	GGAGACGCTG	AGCGACGACG	ACGACGAGGC	GCGGCGCGCG	GCGTCGCTCC
2451	TGAACGGCGC	CGGCATGTCC	GACCCCGCCT	CCGCTAGCAG	CACCAGCACC
2501	AGTAGCAACA	GCGGCACCAG	CAACCTGGTG	GGGCGTGTGT	TCGTGCCCAT
2551	GCGGCTGGTA	TCGGTCAGCC	GCTTCCGCCT	CCGCGACGCT	CTGGGCCGGC
2601	CGCTGCCCGA	CGGCCTGGCC	GTGCCGCCTA	CTGGGCCCAT	CGTGCTCAGC

Fig. 11A

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4901 GAGGAGCCTG AATCCATGGC GCTGCCGCCT CTGGATGCAG TGCGCCGCCC
4951 CATGCCCGAT GCCGACGACA TGGATCTCAT GCGCGGCTCC CACCACCACC
5001 ACCACCACCT CGGCGAGCAG AAGCTGATCT CCGAGGAGGA CCTGGGTAAC
5051 CGATACCCCT ACGACGTGCC CGACTACGCC TACCCCTACG ACGTGCCCGA
5101 CTACGCCGAT CGATCCGGAC CGTACCCCTA CGACGTGCCC GACTACGCCG
5151 CTAGCAGTAC TCGCCGGCCC CCCGACAGGC CTCCCCGTAA CCTGGTCATC
5201 GGAGGAGGTG CCAGGCTTCC GACGGCCGGC GTTAGCGCAT TTGTATGCAT
5251 GAGCGCGGTT GTTTACATGC TGGTGGGCGT GGTGCGCGGG GGGCCGGCGG
```

Fig. 11B

5301 CGTCGGGTGC TGGTGTGGTG TGCTAGTTTT AGCCAGCATA CCGTACACTC
5351 CTGGCGGCCA ACTCGAGTTG TAGCTTGAAT GCATATGCAG ACAGGAAGGC
5401 AGTTGTGTGA GGTGGGGGGC GGACAGCGTA ATGGAGTACC GTCAGTAAGA
5451 GGATGGAGTC ACGGAGCATG TGCTAGAATG TTCCTGAGTG CTGCATGAAC
5501 TGGCTACTGC CTTGGGGAGT CGACCGCGCC TGCGGTGGTT GGGTCAGTCT
5551 CAGCAATTTC AGGTGGGTGA TGAGACGATG AGCTCTAATG CATATTATCG
5601 TATGCGCTGC TGCCTGCGCA CTCTCGCTGT GTGAGAGGAG GGCTACAGTA
5651 GGCTAGGCCG TGTAGGGCGT GCAGGCAACA CAAGAGAGCC AGTCAAGCAG
5701 AGAGAGAGAG GGAGGGGGCA GGTGTTGCAG TGAGAACTGG AATCGTGTCG
5751 CAGACAGATT GCAAGGCGTA GTCCCACGGC GGCTATCTGC ACACAGAGCT
5801 GCCGTGTGAC ATGTGCAAAA GGGCGTGAGG AGATGGCAGT GGCTGTAACA
5851 AGAACAAAGA GCGGCCGCGA ATTCGATATC AAGCTTATCG ATA

Fig. 11C

```
   1 CTCGAGCAGA GGTTGGGAAT CGCTTTGAAA ATCCAGCAAT CGGGTCTCAG
  51 CTGTCTCAGG CCGCACGCGC CTTGGACAAG GCACTTCAGT AACGTACTCC
 101 AAGCCCTCTA TCTGCATGCC CACAAAGCGC AGGAATGCCG ACCATCGTGC
 151 CAGACTGTGC CGCGCCCGAA CCGAAATCCG TCACTCCCCT TGGTTCCCAT
 201 GGTGGCATGG TCCCCCCTGT TCGCCCAAAG CCTGGTTCAG CGCCCAGTGG
 251 CAAACGGCTT TGGCTCAGCT CCTTGGTATT GCTGGTTTCT AGCAATCTCG
 301 TCCGTTCCTC TGTTGCCAAT GTAGCAGGTG CAAACAGTCG AATACGGTTT
 351 TACTCAGGGG CAATCTCAAC TAACAGAGGC CCTGGGCCTG TTGCCTGGAA
 401 CCTATGAAGA CGATAATGCC ACGGCGACTT TCGAGCCTGA GGGAAGTTTG
 451 CACCGGTACC GCATTGTGCA AGGTTACGGT ACATGATAGG GGGAGTGCGA
 501 CGCGGTAAGG CTTGGCGCAG CTTGGCGCGT CTGCCTTGCA TGCATGTCCG
 551 AAACACGCCA CGTCGCGCCA CGAAAAGCGG TAAAAGGACC TGCCATGGTC
 601 CTCCAGGGTG TTACCACTTC CATTTCGCTC AGCTGGGATG GTGCTCGTAG
 651 GTGCACCAGC GTTGATTATT TCAGGCAGGA AGCGGCTGCG AAGCCCGCCT
 701 TTCACTGAAG ACTGGGATGA GCGCACCTGT ACCTGCCAGT ATGGTACCGG
 751 CGCGCTACCG ATGCGTGTAG TAGAGCTTGC TGCCATACAG TAACTCTGGT
 801 ACCCCCAGCC ACCGGGCGTA GCGAGCAGAC TCAATAAGTA TGATGGGTTC
 851 TTATTGCAGC CGCTGTTACA GTTTACAGCG CAAGGGAACA CGCCCCTCAT
 901 TCACAGAACT AACTCAACCT ACTCCATCca tatg CTTCAG TTGGCGAACC
 951 GTAGCGTGAG GGCTAAGGCC GCTCGTGCCA GCCAAAGCGC TCGGAGTGTC
1001 TCGTGTGCGG CTGCCAAGCG CGGTGCGGAT GTTGCTCCCC TGACGTCGGC
1051 CCTGGCGGTC ACCGCATCCA TCCTGCTCAC GACTGGCGCG GCGAGCGCTA
1101 GCGCAGCTGA CCTCGCTCTC GGCGCCCAGG TCTTCAACGG CAACTGTGGT
1151 GAGTAGCTCA TGCAAATTTA GCATGATCGA AGGCTGCGCG TGTCATGGGT
1201 CTCCGCTCGC TGTTCGACAT GCCGTTTCGC TCAACTGCAC CATCGACTAT
1251 CGGTCCCCCT CCTTCCACTT CTGGCCCACG CAGCCGCGTG CCACATGGGC
1301 GGTCGCAACA GCGTGATGCC CGAGAAGACG CTGGACAAGG CCGCCCTTGA
1351 GCAGTACCTG GATGGCGGCT TCAAGGTGGA GAGCATCATC TATCAGGTCG
1401 GGACATCCCC GACCAGGGGC GGCGGGGATG TTGCTGGGCC GATGGAAAGT
1451 AGCAACCCAG CCAGCGGCTT CCAGCGCACT CCAGCTGCTC ACGGTTGCGA
1501 CATTGCGCGT GCACGCTTGC GCGTCCCTCA CTCGGCCAGC TTGTCGCCGC
1551 AGACATCCCT AGCATTGTGC GGACTGCGGT CGTCAGTTAG CGTAGTGGCG
1601 GGGCTCAAAG CGTGATGCAG CTGGTGGCTG ATTGCATGTG CTACATATGC
1651 TGTTATGTTT TGCATGAACT TCGATGCATT GGATGCTGGG TGCACGCGTT
1701 TGCATGTGTT TGTGCCGGCA TGCTGCCGTC GTCGGCCGTA CGTTTACGTT
1751 TCTGTGTGCC GGGGTCTTTA TTTCCGCCTG CAGGTGGAGA ATGGCAAGGG
1801 GGCGATGCCG GCGTGGGCGG ATCGGCTGTC GGAGGAGGAA ATCCAGGCTG
1851 TGGCGGAGTA CGTGTTCAAG CAGGCCACGG ATGCCGCCTG GAAGTACTAG
1901 GTTGATGTTG TTATTTCAAC TGGGTCACCG TAGCTAGCTC GTGCCCCAGT
1951 TGTGGATGCG AGTTATACGT CATTGCGTAA CATGTTCATG ATAGACTGCA
2001 TTAGGTAGGC GTCGTGTGTG AGCACATACA GAAGTCATCA CGCAAATGGA
2051 CACGTTCCGG CGAACCCGAG GGGAAAGGCT TGGGCCAGTA CATTATTTCA
2101 ACACTAAAAT ATGTAACATA ATGGAACTTG AGCACGGTCC GGGAGCGCAG
2151 GCTGGGCTTG GGGGTCGCGG CTCGAGGGAG AGGGGCGACG TTGGGGCAGG
2201 TCGGGGCTTC AACCGGGTTT TGCACGGCCG AACCATGAAC GCGCTTTGGC
2251 CAGCCAAGAT ACTGAAAATA CAACAGAAGG ATATCCAGTA TGTAGCAAAG
2301 CCTTCAAACA GCGTGTACAA GCAAGCCTGT GACAAAGCGG ACCCGGCCGT
2351 GAAGTCCACG GTATTTCCTC AAGCAGCATT CAGATGAGAG AAGGAATGGG
2401 CTCTCCATCT GTTTACATTC AGTCGCATTC CACTTGTCCT GGCGCATCGT
2451 CTGTCGCTAG ACGTCGCCGC TCAAAGCGTT TTCGCGGTGG CAGCACCGGC
2501 TAAGAACCGA AGGCGATCGC AGTCCATTTT CCTGACGTTG GACGCTTTGA
2551 GGGCACGAGG CGATGGCTGC GGGCTGCGGG CTGCATGGTT GTTTCCGGAG
2601 CAGAGTC
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Fig. 12

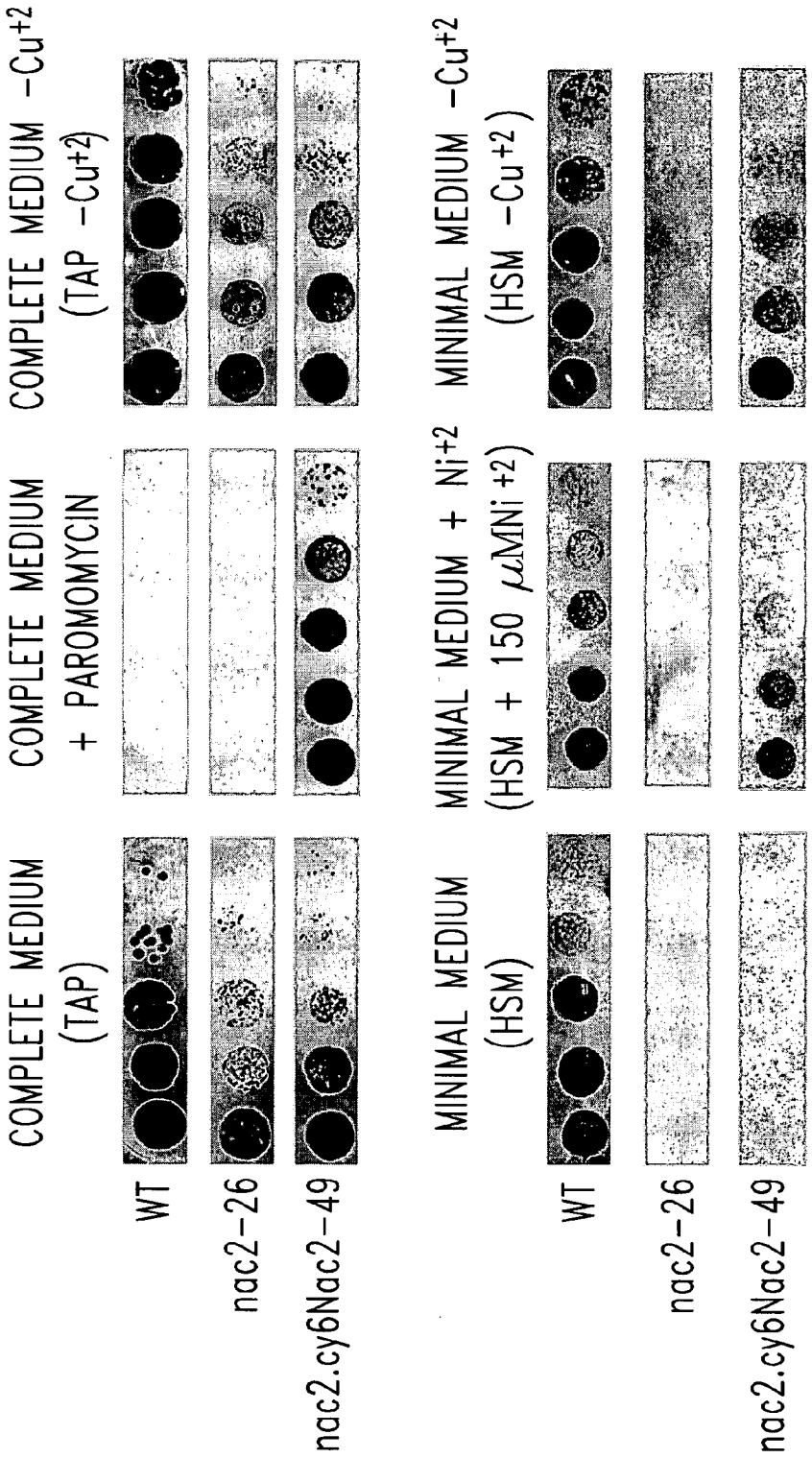

COMPLETE MEDIUM (TAP)
COMPLETE MEDIUM + PAROMOMYCIN
COMPLETE MEDIUM −Cu+2 (TAP −Cu+2)
MINIMAL MEDIUM (HSM)
MINIMAL MEDIUM + Ni+2 (HSM + 150 μMNi+2)
MINIMAL MEDIUM −Cu+2 (HSM −Cu+2)
WT
nac2-26
nac2.cy6Nac2-49

Fig. 13

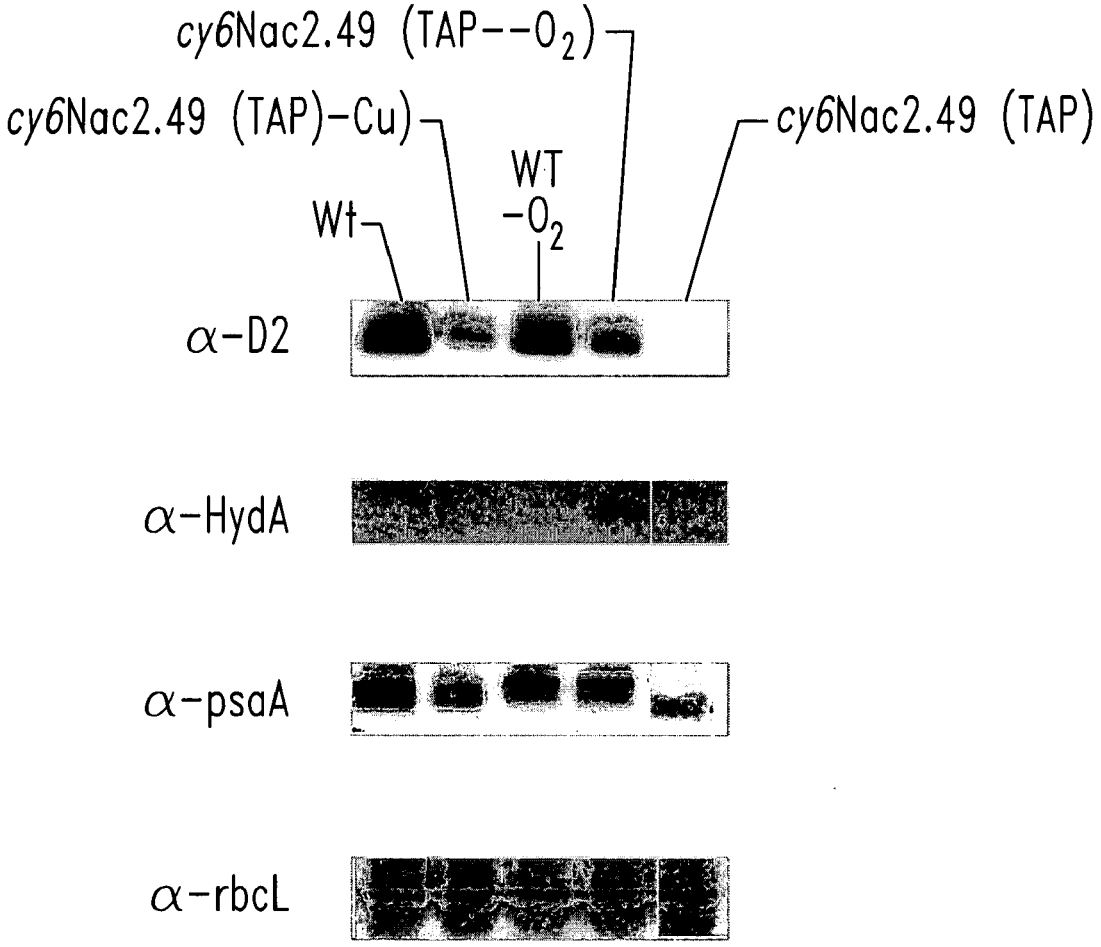

cy6Nac2.49 (TAP--O2)
cy6Nac2.49 (TAP)-Cu)
cy6Nac2.49 (TAP)
WT
-O2
Wt
α-D2
α-HydA
α-psaA
α-rbcL

Fig. 14

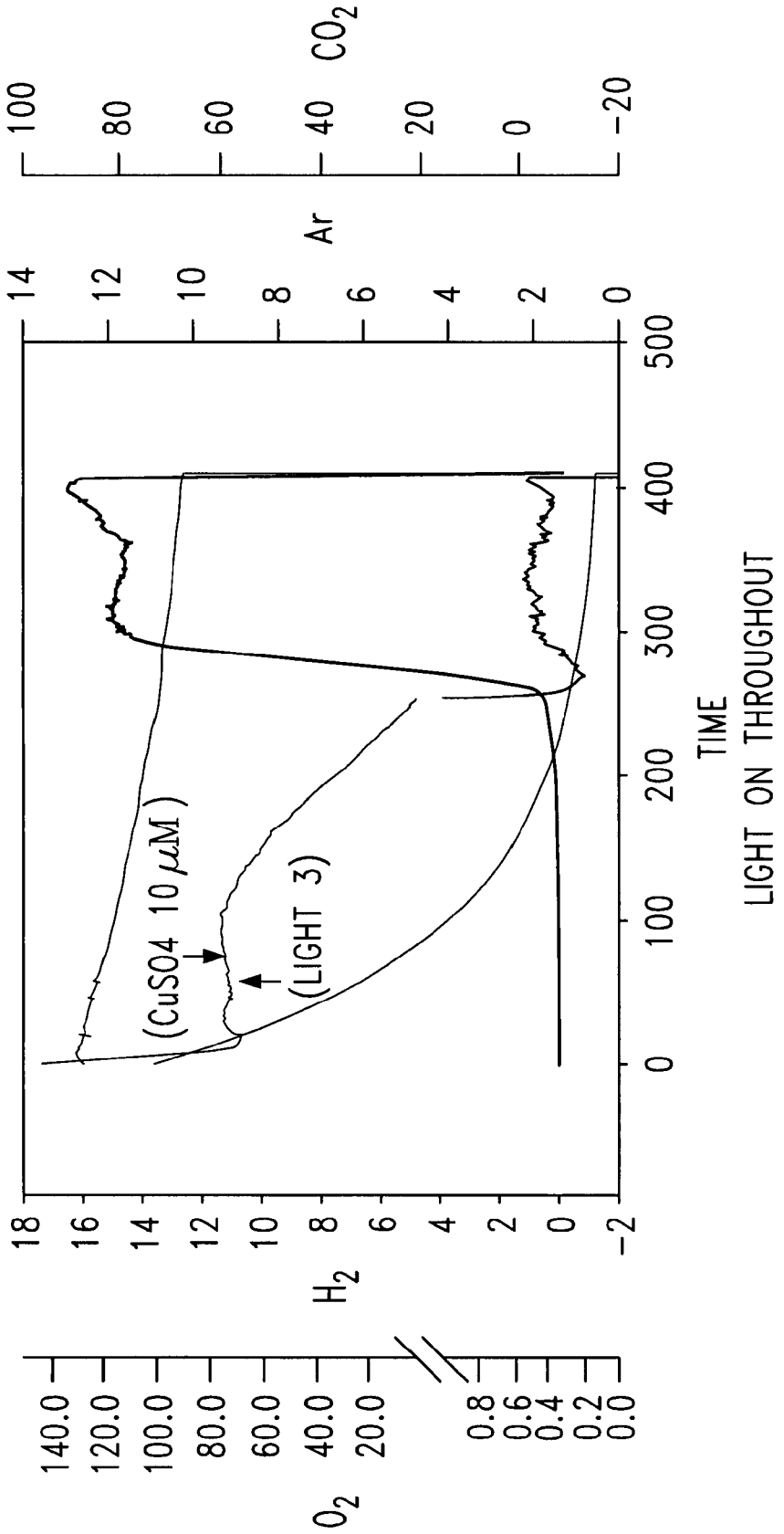

O_2
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.8
0.6
0.4
0.2
0.0
H_2
18
16
14
12
10
8
6
4
2
0
-2
Ar
14
12
10
8
6
4
2
0
CO_2
100
80
60
40
20
0
-20
0
100
200
300
400
500
TIME
LIGHT ON THROUGHOUT
(CuSO4 10 μM)
(LIGHT 3)

Fig. 15

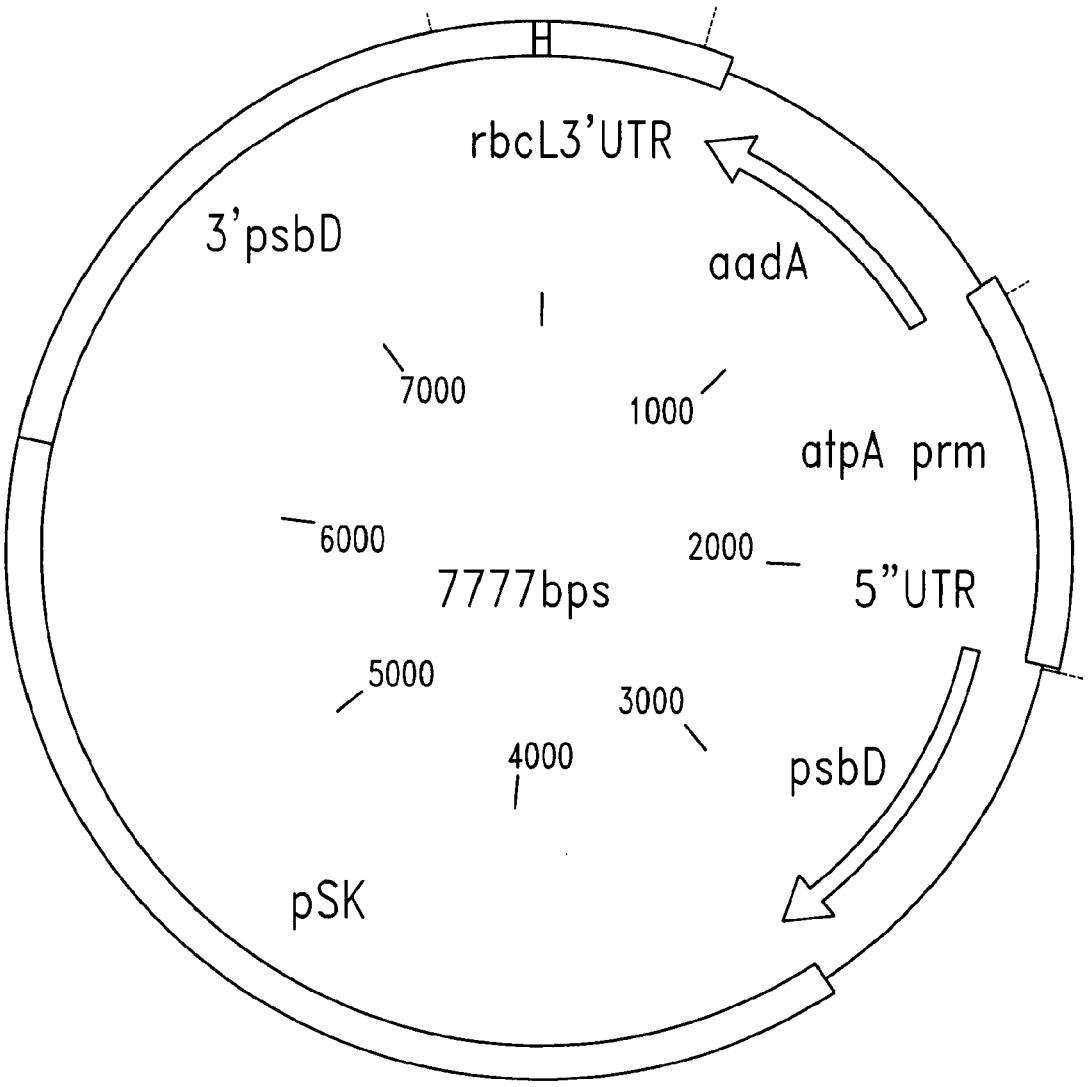

rbcL3'UTR
3'psbD
aadA
1
7000
1000
atpA prm
6000
2000
7777bps
5"UTR
5000
3000
4000
psbD
pSK

Fig. 16

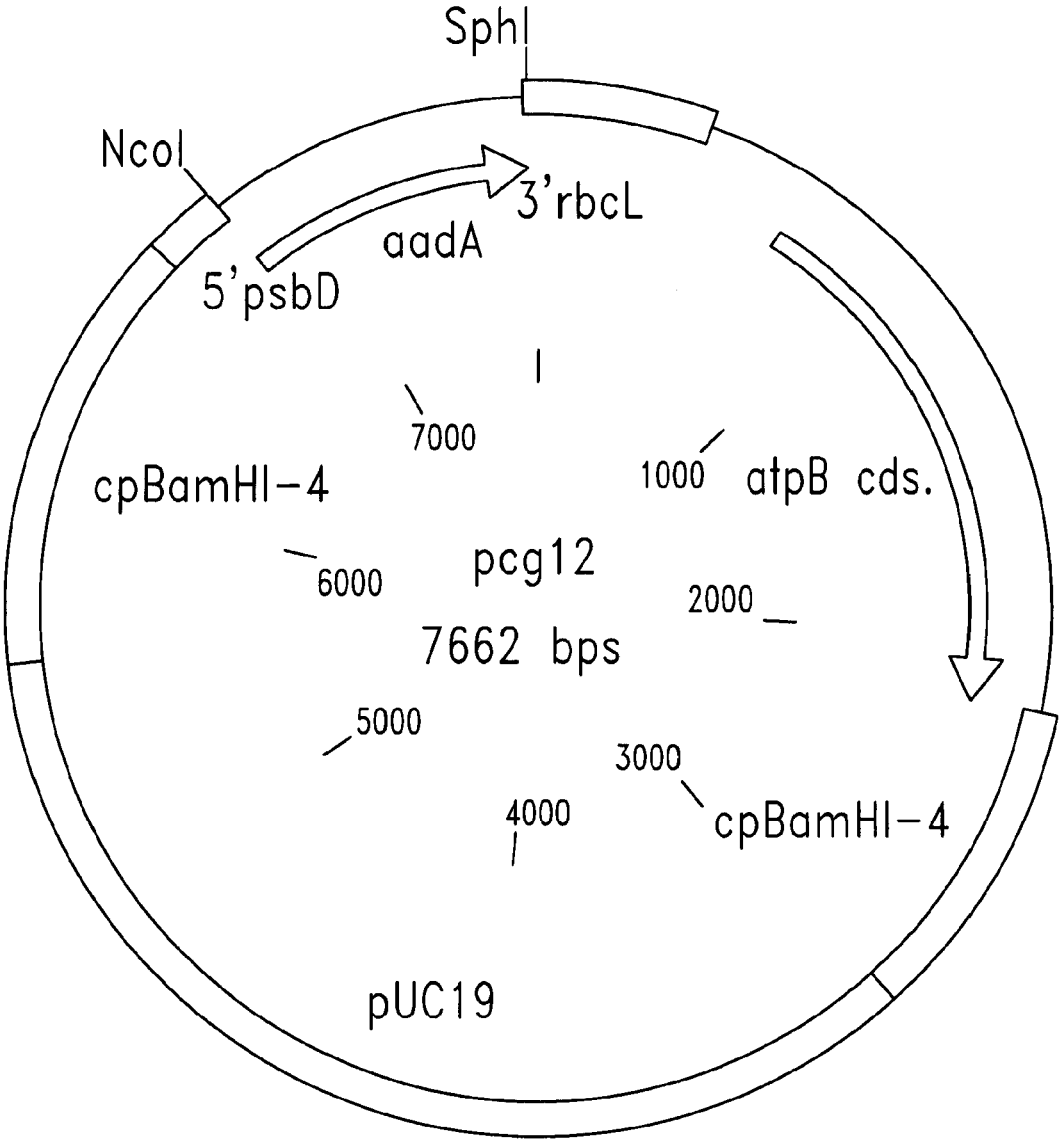
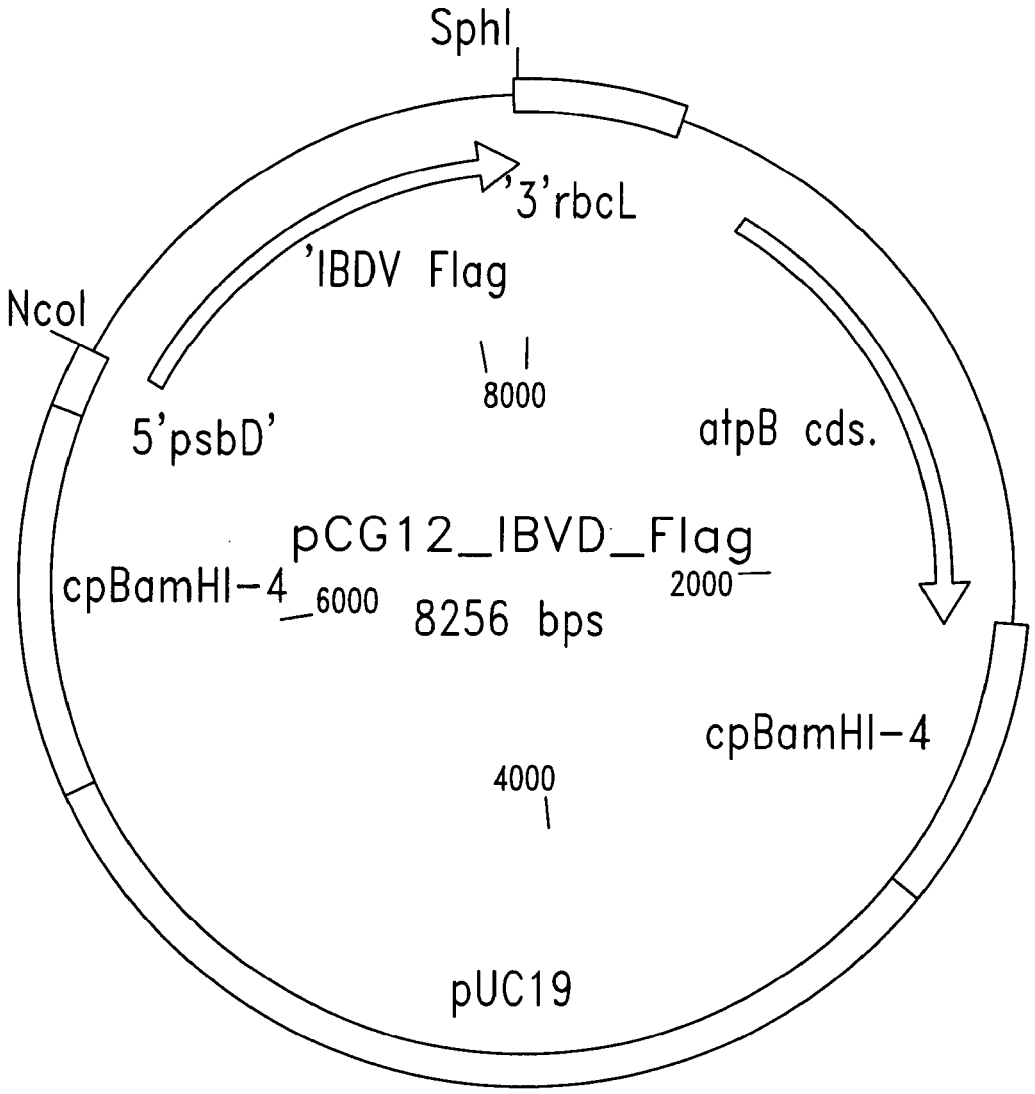

SphI
NcoI
3'rbcL
aadA
5'psbD
7000
1000
atpB cds.
cpBamHI-4
6000
pcg12
2000
7662 bps
5000
3000
4000
cpBamHI-4
pUC19

Fig. 17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'IBDV Flag
NcoI
8000
5'psbD'
atpB cds.
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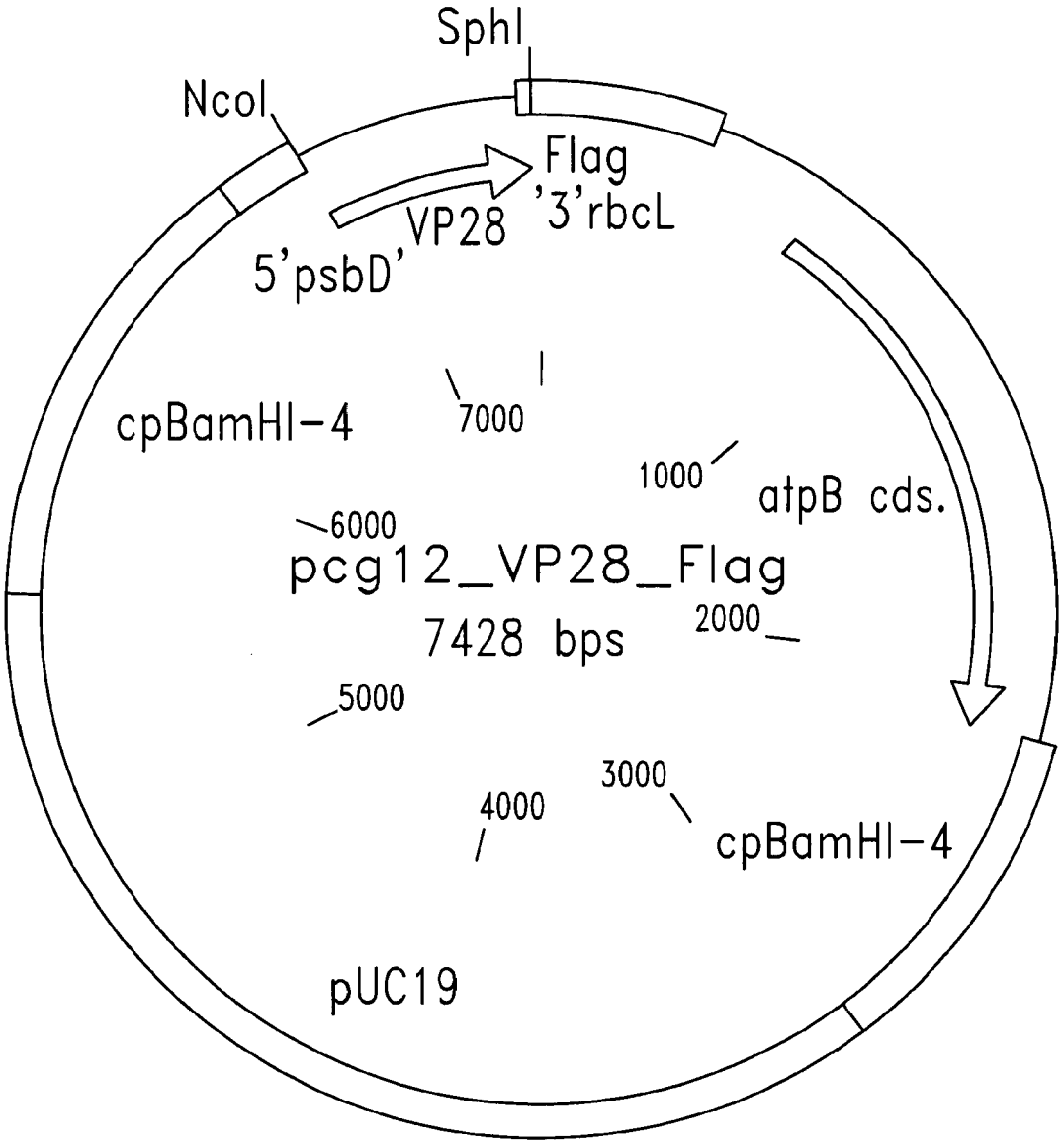
6000
8256 bps
2000
cpBamHI-4
4000
pUC19

Fig. 18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NcoI
Flag
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VP28
5'psbD'
cpBamHI-4
7000
1000
atpB cds.
6000
pcg12_VP28_Flag
7428 bps
2000
5000
3000
4000
cpBamHI-4
pUC19

Fig. 19

1	GAGTCATATG	AAATTAAATG	GATATTTGGT	ACATTTAATT	CCACAAAAAT
51	GTCCAATACT	TAAAATACAA	AATTAAAAGT	ATTAGTTGTA	AACTTGACTA
101	ACATTTTAAA	TTTTAAATTT	TTTCCTAATT	ATATATTTTA	CTTGCAAAAT
151	TTATAAAAAT	TTTATGCATT	TTTATATCAT	AATAATAAAA	CCTTTATTCA
201	TGGTTTATAA	TATAATAATT	GTGATGACTA	TGCACAAAGC	AGTTCTAGTC
251	CCATATATAT	AACTATATAT	AACCCGTTTA	AAGATTTATT	TAAAAATATG
301	TGTGTAAAAA	ATGCTTATTT	TTAATTTTAT	TTTATATAAG	TTATAATATT
351	AAATACACAA	TGATTAAAAT	TAAATAATAA	TAAATTTAAC	GTAACGATGA
401	GTTGTTTTTT	TATTTTGGAG	ATACACGCAA	TGACAATTGC	GATCGGTACA
451	TATCAAGAGA	AACGCACATG	GTTCGATGAC	GCTGATGACT	GGCTTCGTCA
501	AGACCGTTTC	GTATTCGTAG	GTTGGTCAGG	TTTATTACTA	TTCCCTTGTG
551	CTTACTTTGC	ATTAGGTGGT	TGGTTAACTG	GTACTACTTT	CGTTACTTCA
601	TGGTATACGC	ATGGTTTAGC	TACTTCTTAC	TTAGAAGGTT	GTAACTTCT
651	AACAGCAGCT	GTTTCTACAC	CTGCTAACAG	TATGGCTCAC	TCTCTTCTAT
701	TTGTTTGGGG	TCCAGAAGCT	CAAGGTGATT	TCACTCGTTG	GTGTCAACTT
751	GGTGGTTTAT	GGGCATTCGT	TGCTTTACAC	GGTGCATTTG	GTTTAATTGG
801	TTTCATGCTT	CGTCAGTTTG	AAATTGCTCG	TTCAGTAAAC	TTACGTCCAT
851	ACAACGCAAT	TGCTTTCTCA	GCACCAATTG	CTGTATTCGT	TTCAGTATTC
901	CTAATTTACC	CATTAGGTCA	ATCAGGTTGG	TTCTTTGCAC	CTAGTTTCGG
951	TGTAGCTGCT	ATCTTCCGTT	TCATTTTATT	CTTCCAAGGT	TTCCACAACT
1001	GGACACTTAA	CCCATTCCAC	ATGATGGGTG	TTGCTGGTGT	TTTAGGTGCT
1051	GCTTTATTAT	GTGCTATTCA	CGGTGCTACT	GTTGAAAACA	CATTATTCGA
1101	AGACGGTGAC	GGTGCTAACA	CATTCCGTGC	ATTCAACCCT	ACACAGGCTG
1151	AAGAAACATA	CTCTATGGTT	ACTGCTAACC	GTTTCTGGTC	ACAAATCTTC
1201	GGTGTTGCTT	TCTCTAACAA	ACGTTGGCTT	CACTTCTTCA	TGTTATTAGT
1251	TCCAGTAACT	GGTCTTTGGA	TGAGTGCTAT	TGGTGTTGTA	GGTTTAGCTC
1301	TAAACTTACG	TGCTTACGAC	TTCGTATCAC	AAGAGATTCG	TGCTGCTGAA
1351	GACCCTGAAT	TCGAAACATT	CTACACTAAA	AACATTCTTC	TTAACGAAGG
1401	TATTCGTGCT	TGGATGGCTG	CTCAAGACCA	ACCACACGAA	CGTTTAGTAT
1451	TCCCTGAAGA	AGTATTACCA	CGTGGTAACG	CTCTATAATA	TATTTTTATA
1501	TAAATTACCA	ATACTAATTA	GTATTGGTAA	TTTATATTAC	TTTATTATTT
1551	AAAAGAAAAT	GCCCCTTTGG	GGCTAAAAAT	CACATGAGTG	CTTGAGCCGT
1601	ATGCGAAAAA	ACTCGCATGT	ACGGTTCTTT	AGGAGGATTT	AAAATATTAA
1651	AAAATAAAAA	AACAAATCCT	ACCTGACTAA	ACCAGGACAT	TTTTCACGTA
1701	CTCTGTCAAA	AGGTCC			

Fig. 20

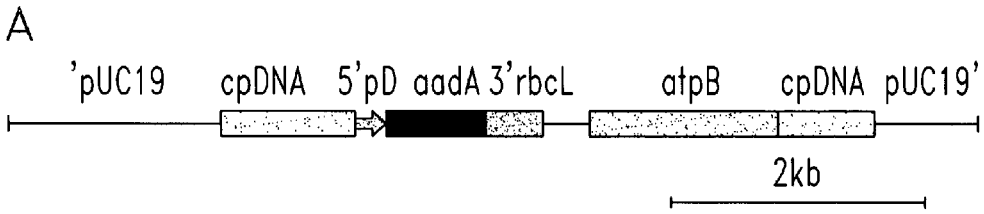


Fig. 21A

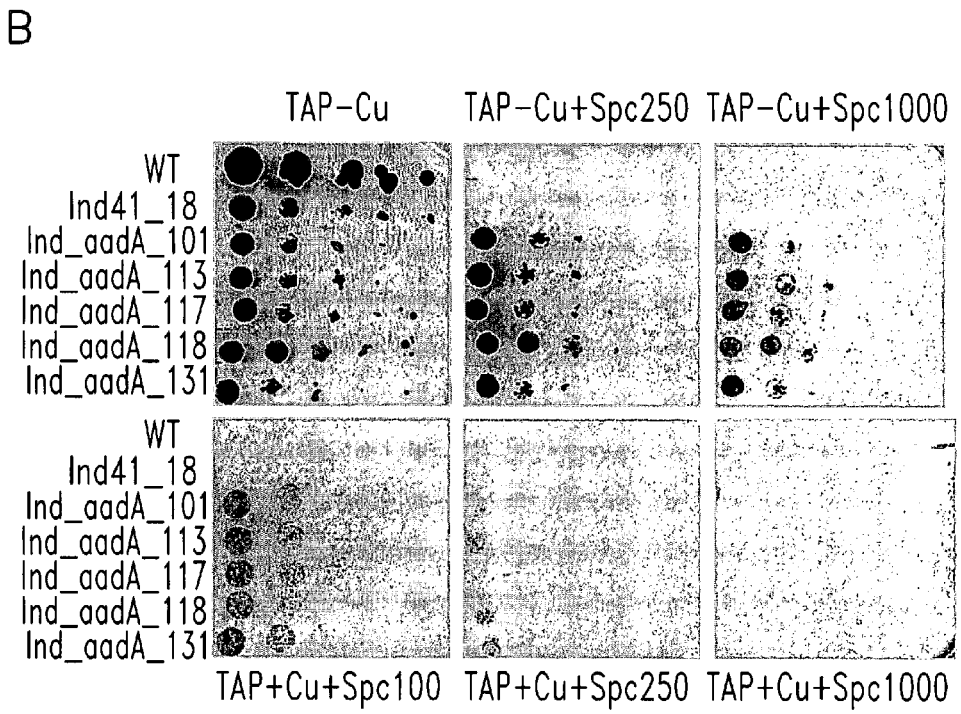


Fig. 21B

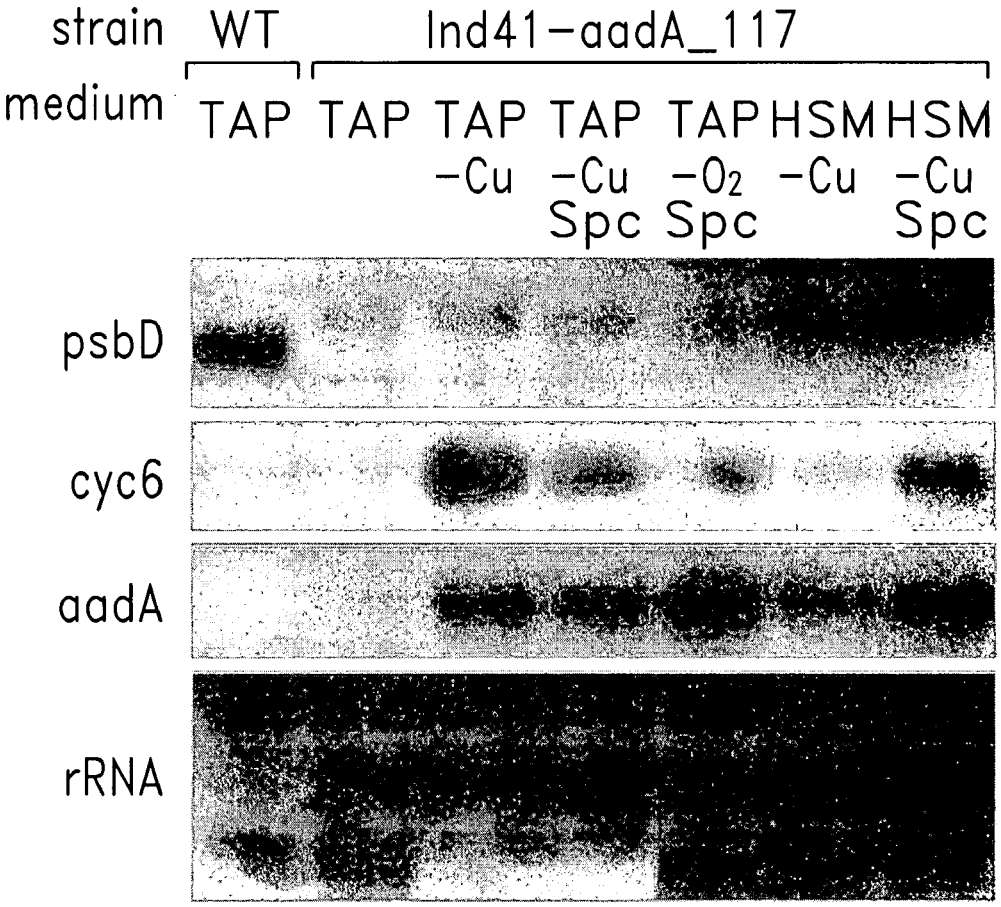
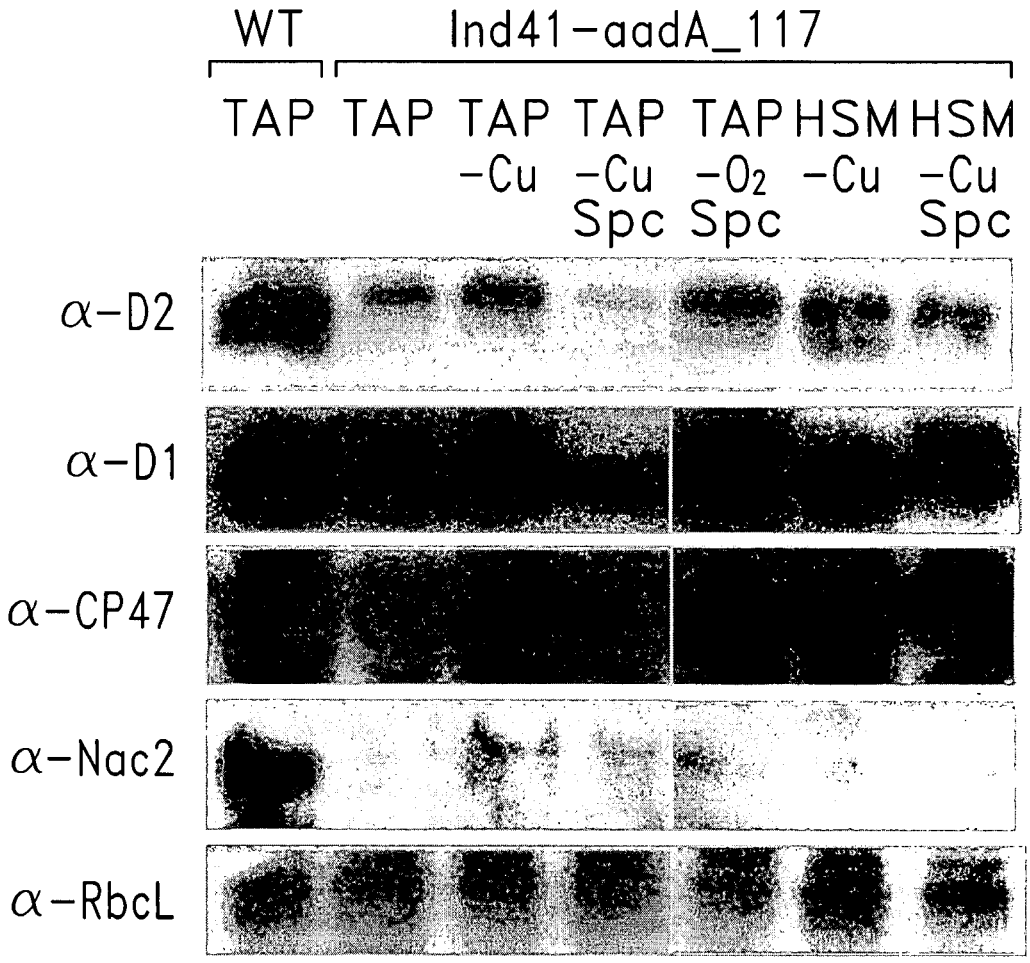

strain
WT
Ind41-aadA_117
medium
TAP
TAP
TAP -Cu
TAP -Cu Spc
TAP -O2 Spc
HSM -Cu
HSM -Cu Spc
psbD
cyc6
aadA
rRNA

Fig. 22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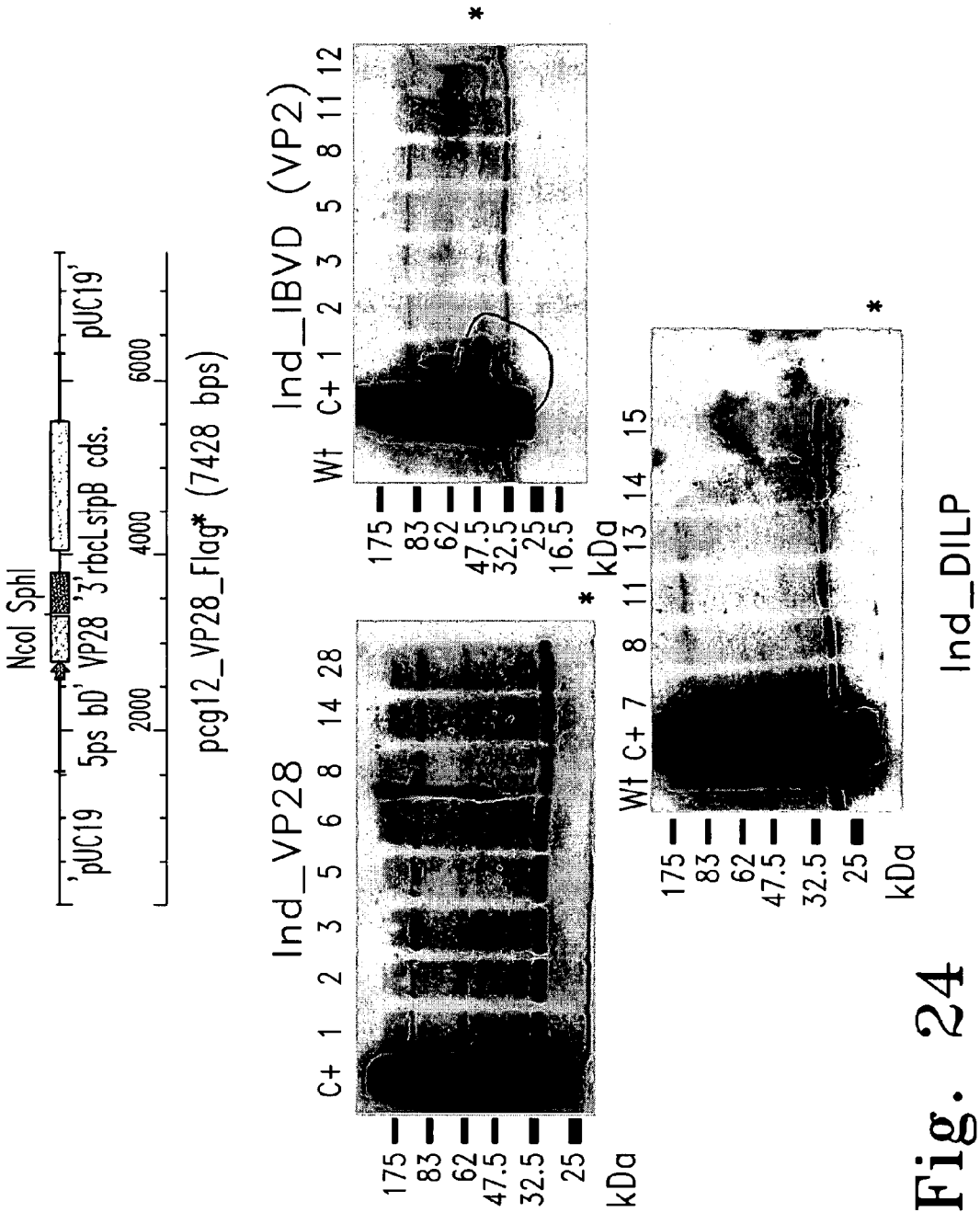
Ind41-aadA_117
TAP
TAP
TAP -Cu
TAP -Cu Spc
TAP -O2 Spc
HSM -Cu
HSM -Cu Spc
α-D2
α-D1
α-CP47
α-Nac2
α-RbcL

Fig. 23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'pUC19 5ps bD' VP28 '3'rbcLstpB cds. pUC19'
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4000
6000
pcg12_VP28_Flag* (7428 bps)
Ind_VP28
C+ 1 2 3 5 6 8 14 28
175
83
62
47.5
32.5
25
kDa
Ind_IBVD (VP2)
Wt C+ 1 2 3 5 8 11 12
175
83
62
47.5
32.5
25
16.5
kDa
Wt C+ 7 8 11 13 14 15
175
83
62
47.5
32.5
25
kDa
Ind_DILP

Fig. 24

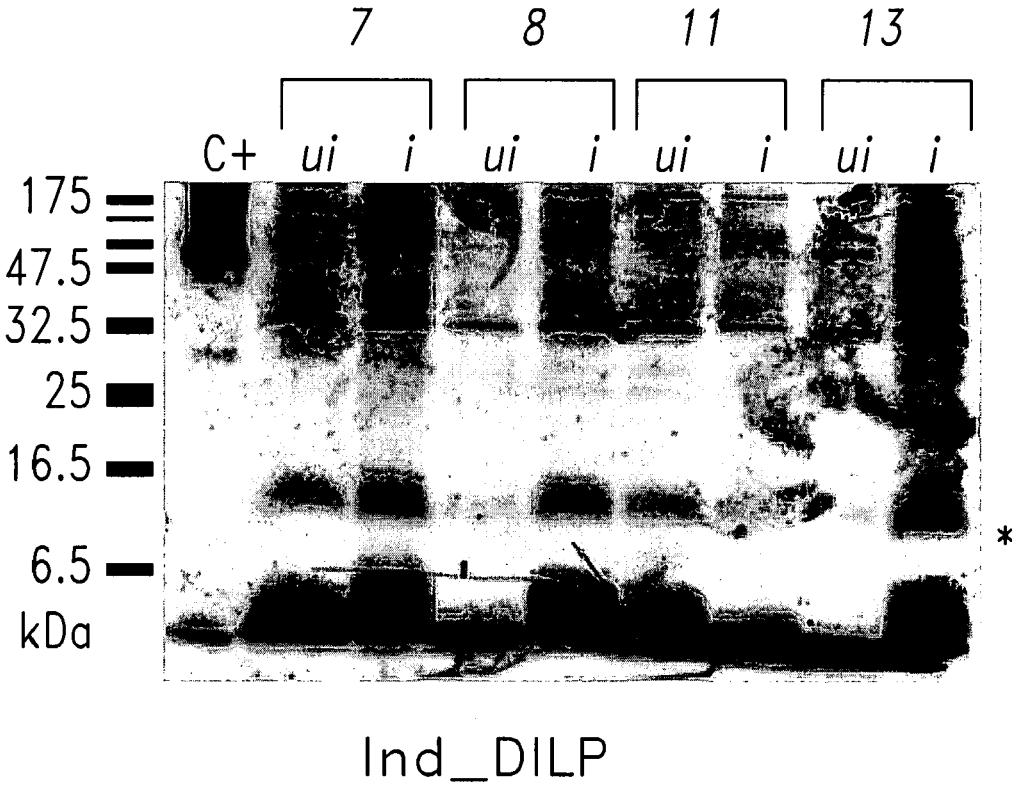


Fig. 25

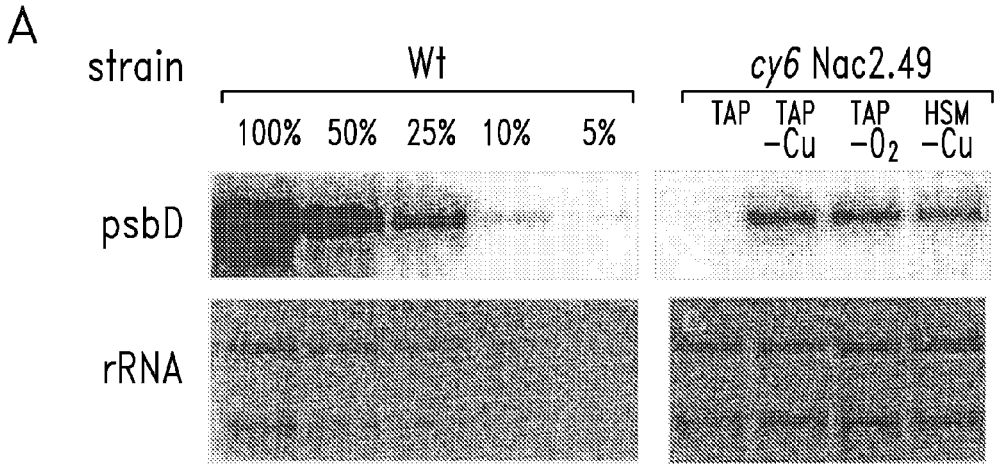


Fig. 26A

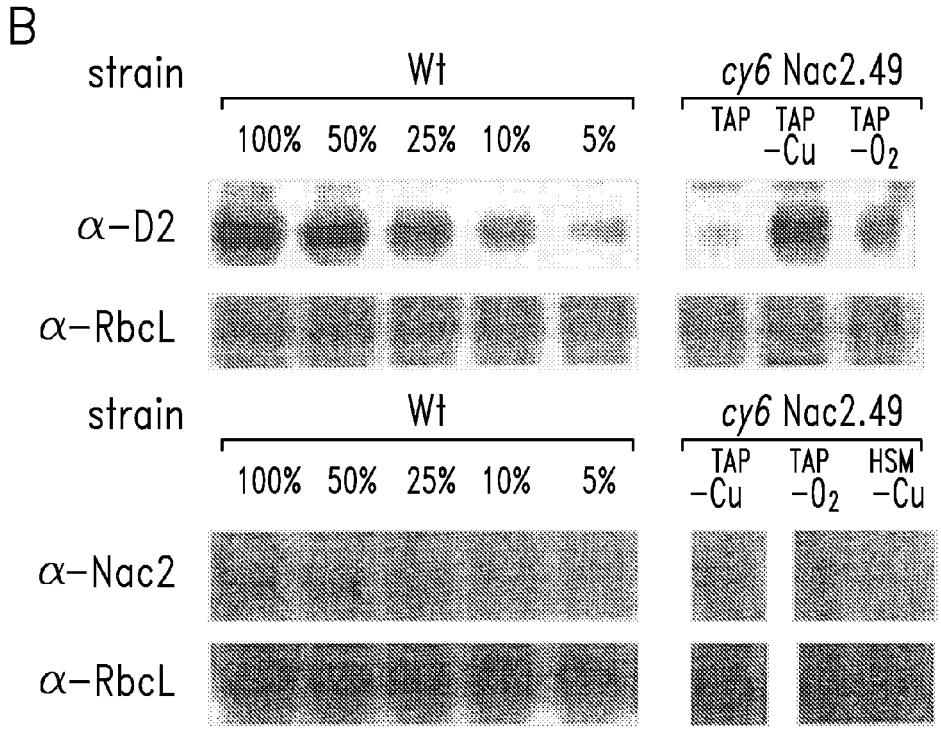


Fig. 26B

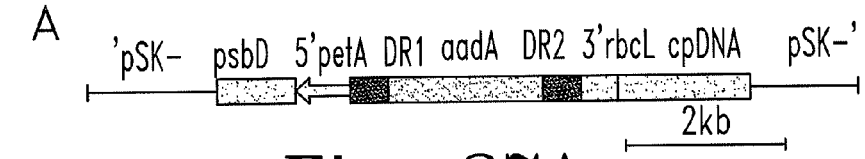


Fig. 27A

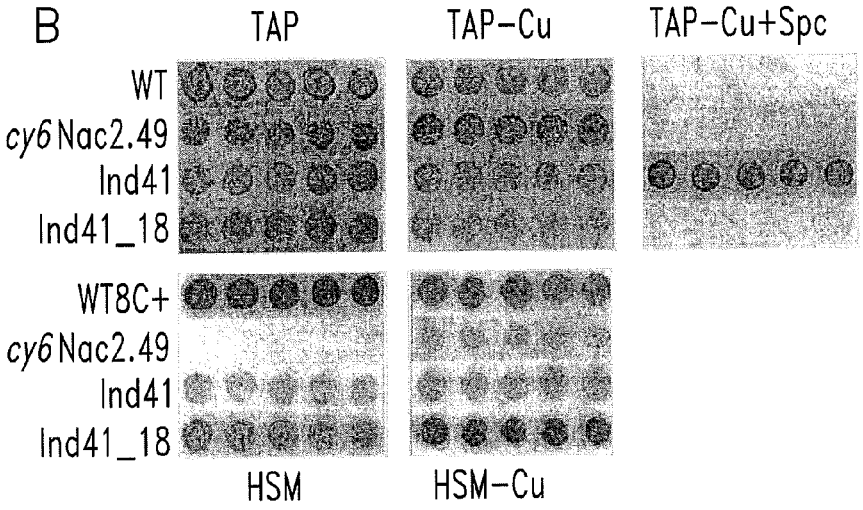


Fig. 27B

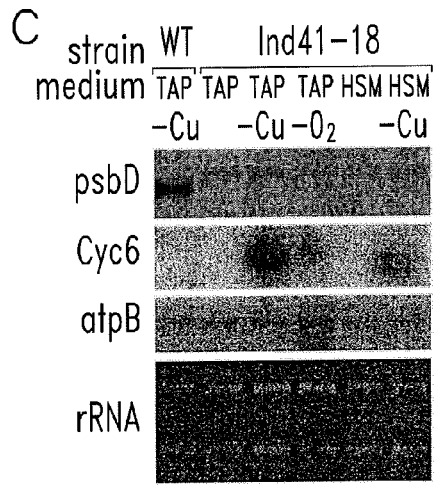


Fig. 27C

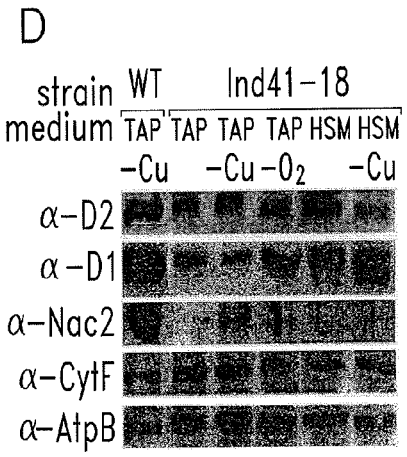


Fig. 27D

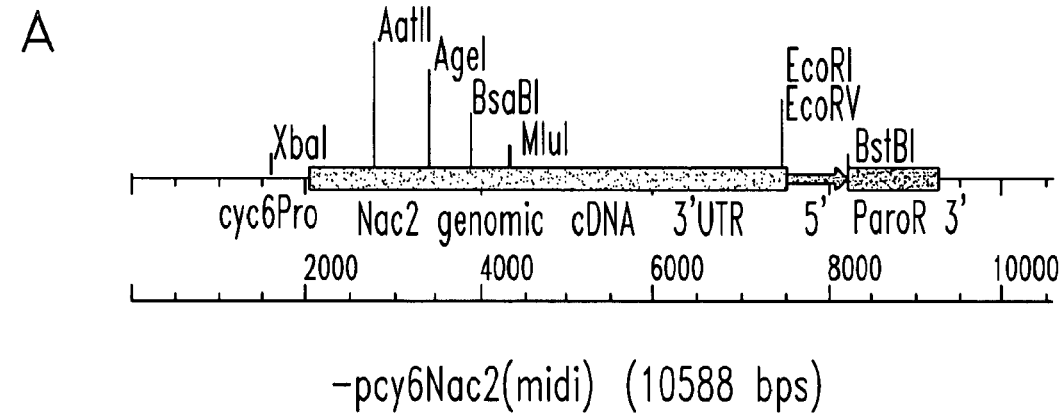


Fig. 28A

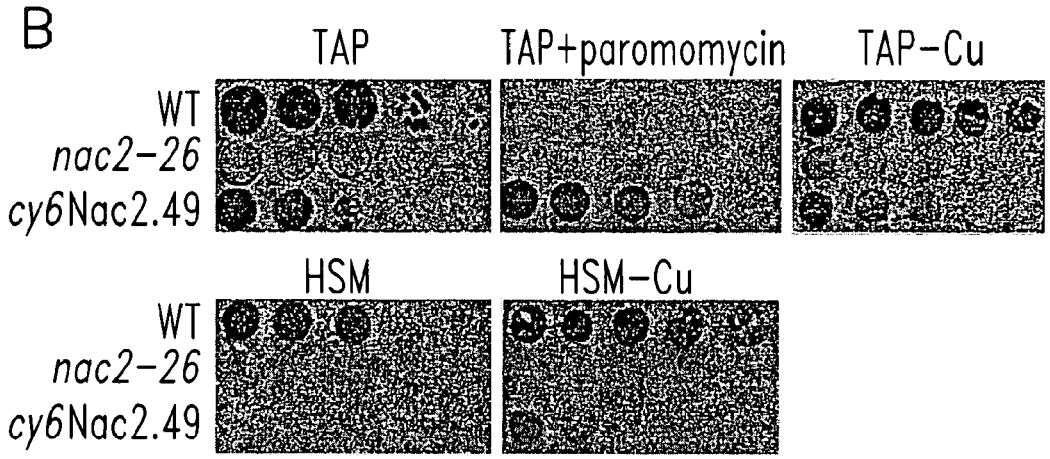


Fig. 28B

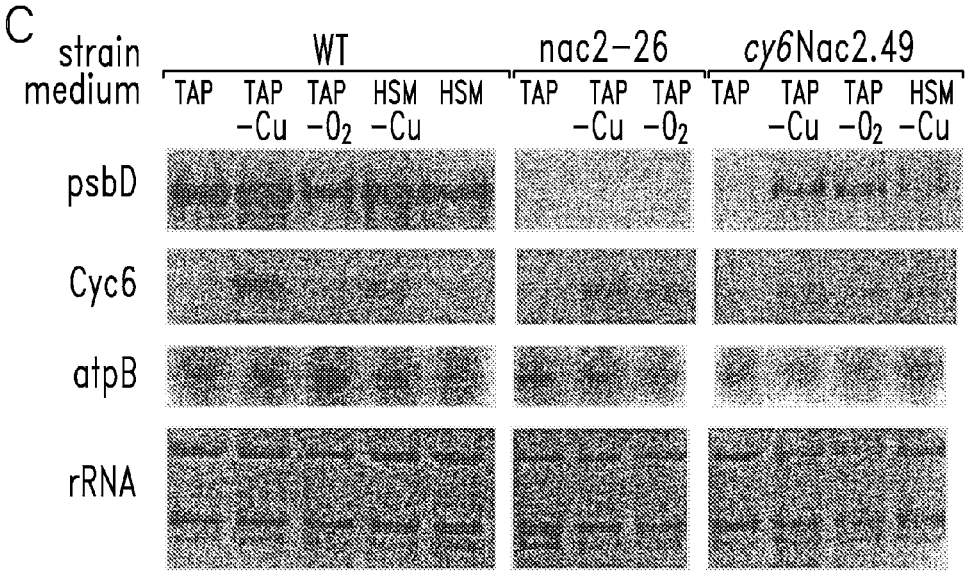


Fig. 28C

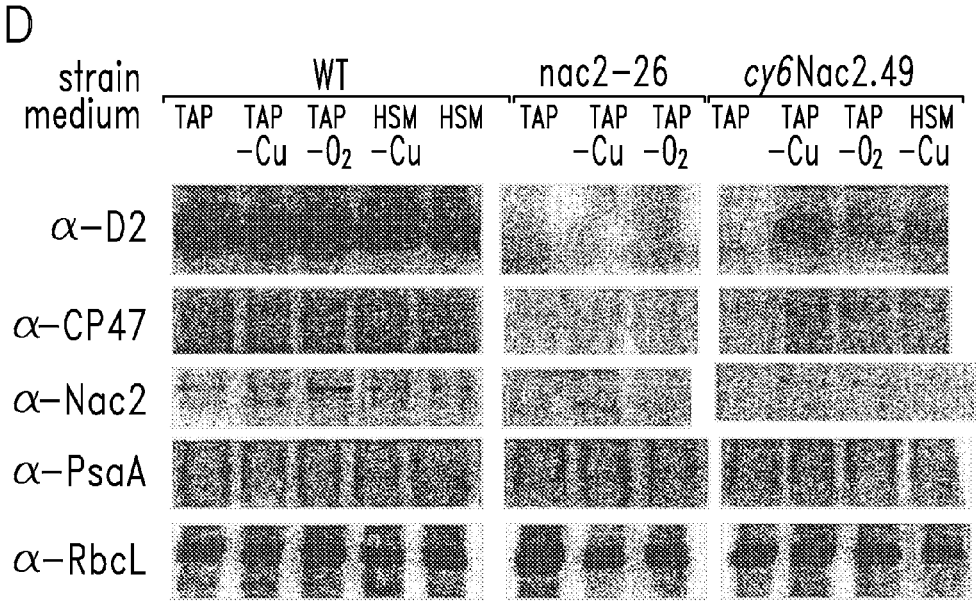


Fig. 28D

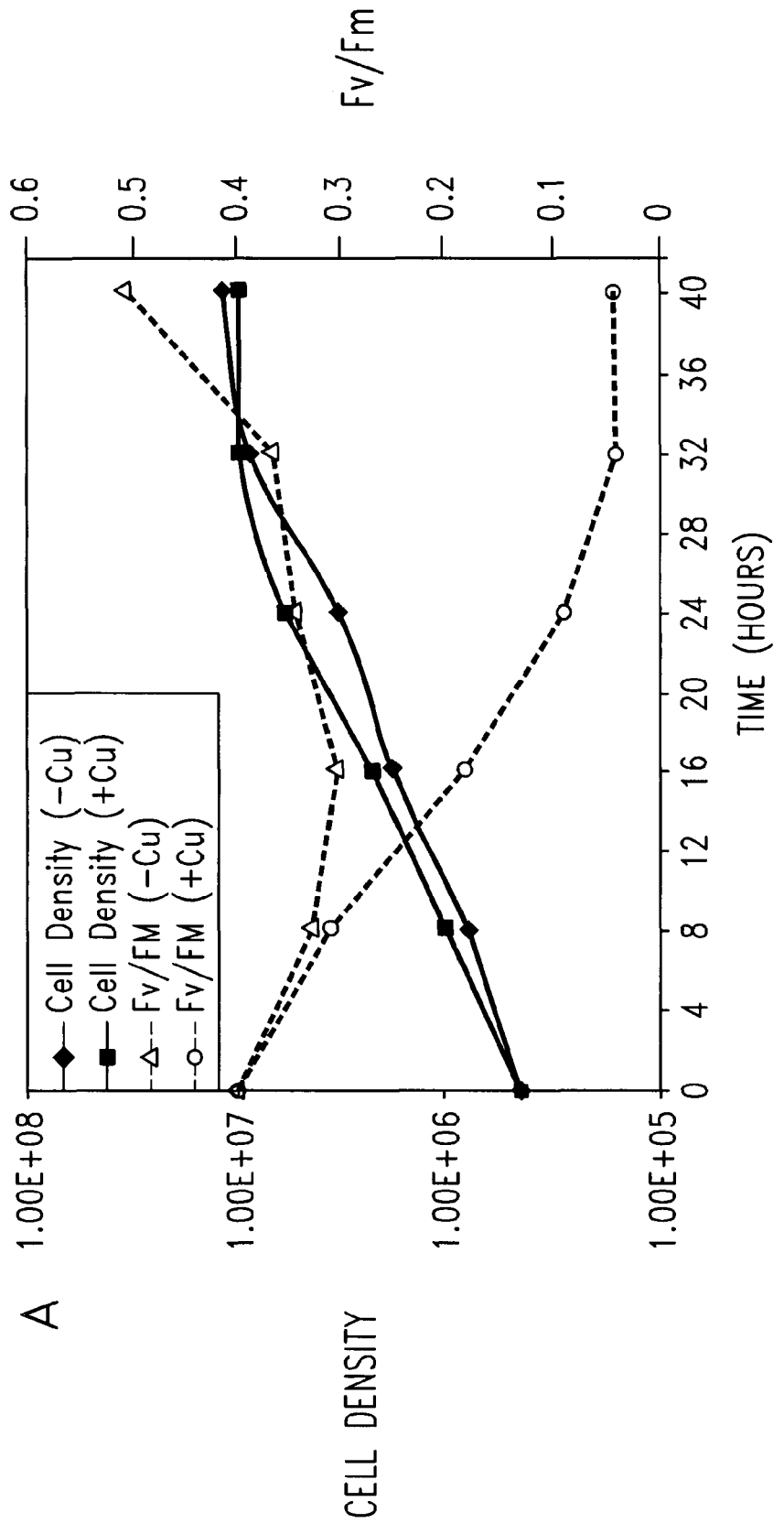

A
CELL DENSITY
1.00E+08
1.00E+07
1.00E+06
1.00E+05
0
4
8
12
16
20
24
28
32
36
40
TIME (HOURS)
0.6
0.5
0.4
0.3
0.2
0.1
0
Fv/Fm
Cell Density (-Cu)
Cell Density (+Cu)
Fv/FM (-Cu)
Fv/FM (+Cu)

Fig. 29A

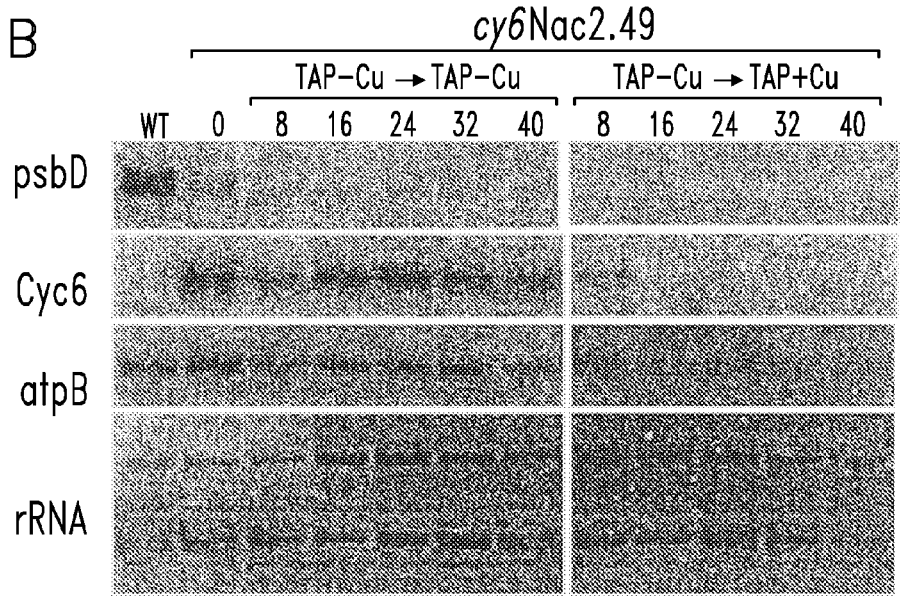


Fig. 29B

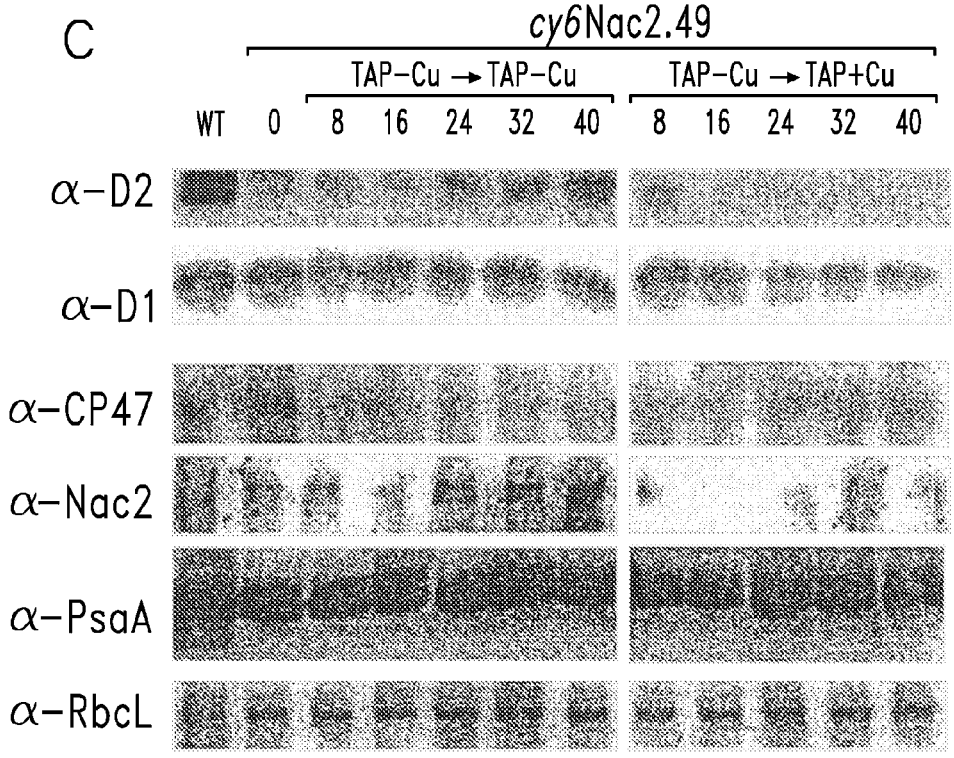


Fig. 29C

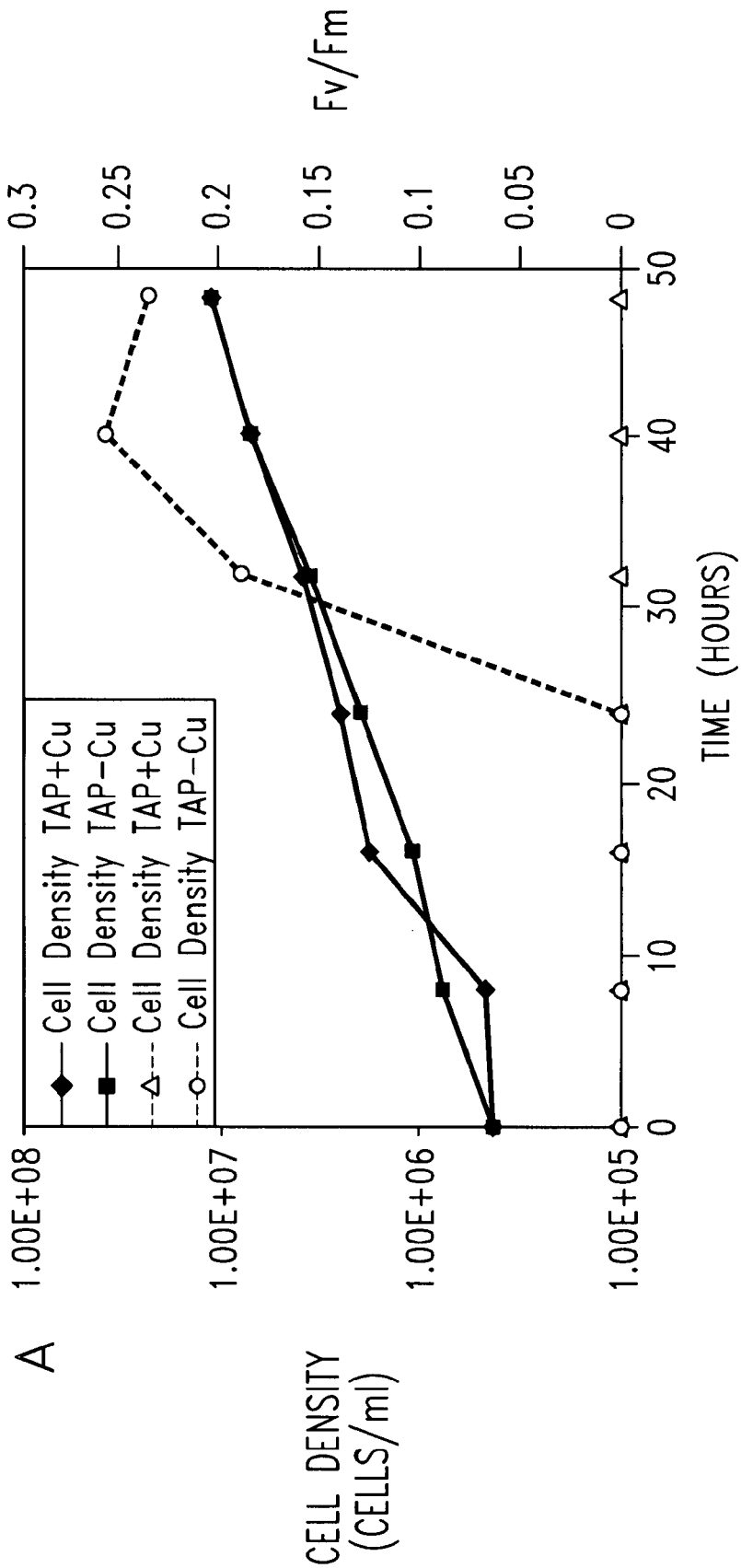

A
CELL DENSITY (CELLS/ml)
1.00E+08
1.00E+07
1.00E+06
1.00E+05
0
10
20
30
40
50
TIME (HOURS)
Fv/Fm
0.3
0.25
0.2
0.15
0.1
0.05
0
Cell Density TAP+Cu
Cell Density TAP−Cu
Cell Density TAP+Cu
Cell Density TAP−Cu

Fig. 30A

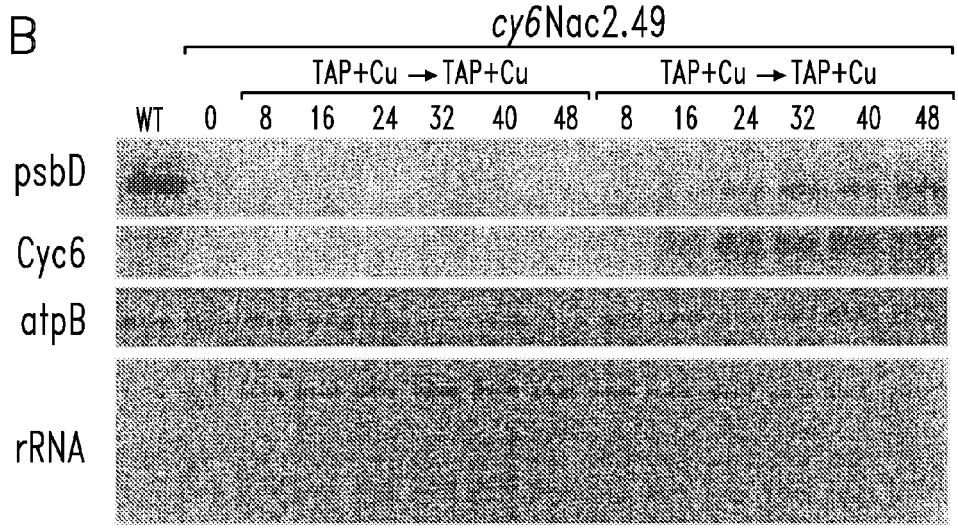


Fig. 30B

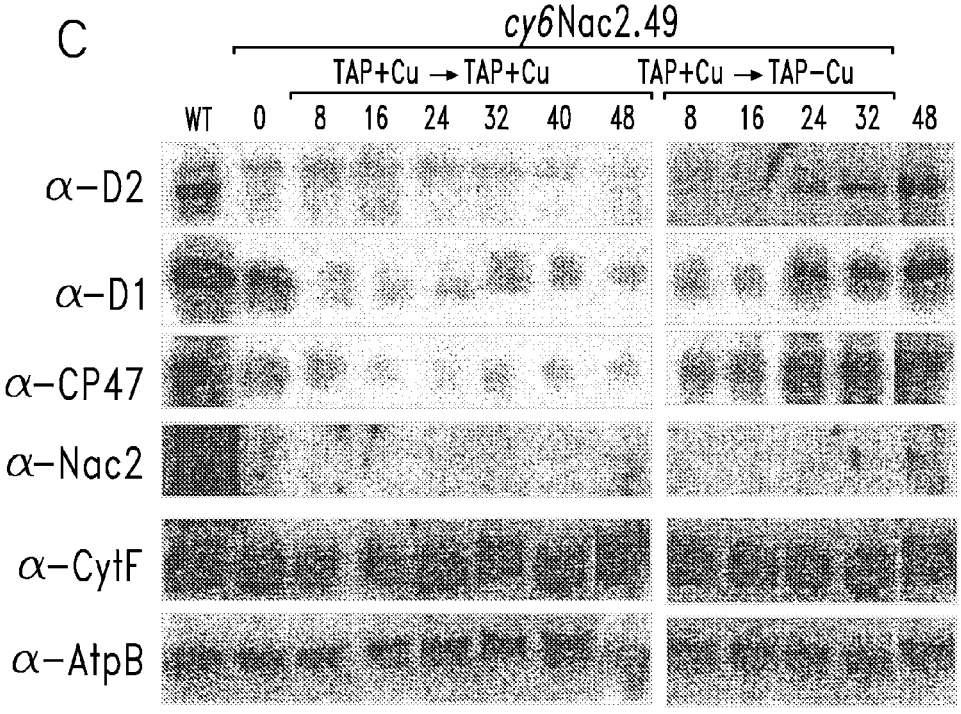


Fig. 30C

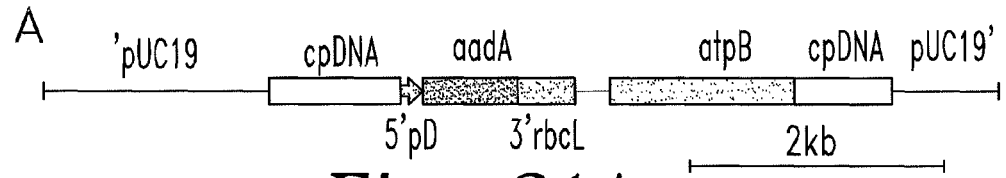


Fig. 31A

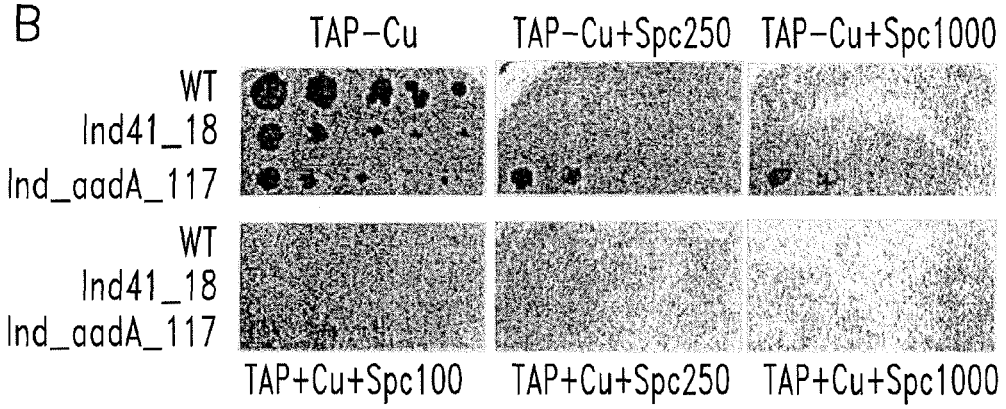
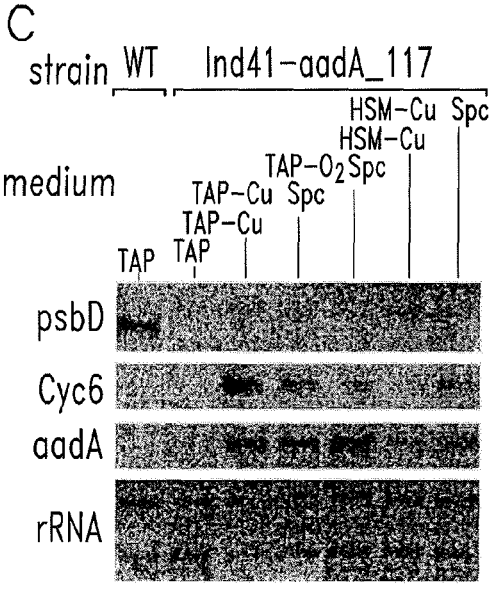


Fig. 31B



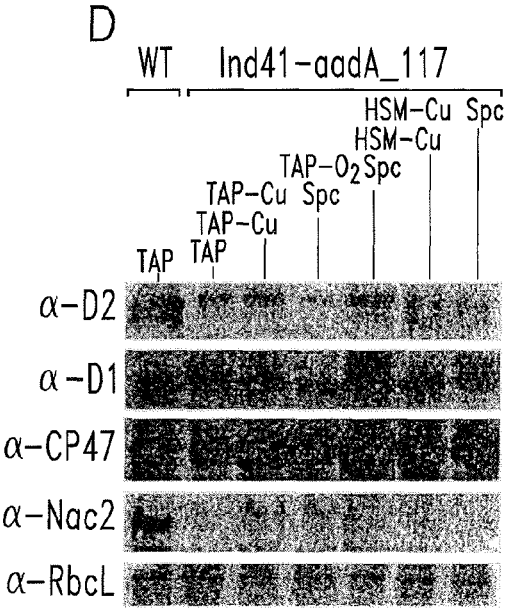


Fig. 31C Fig. 31D

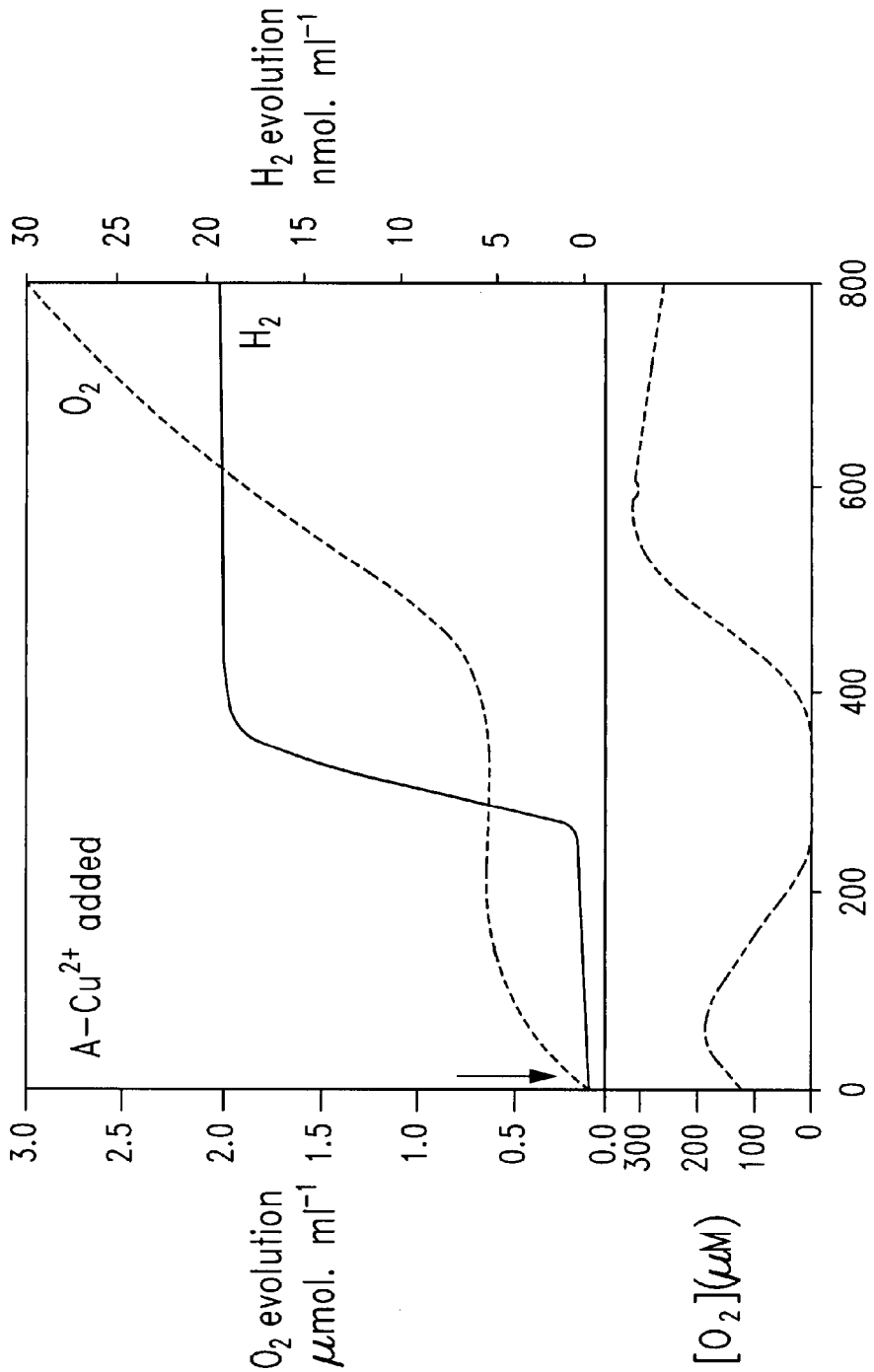

H_2 evolution nmol. ml^{-1}
30
25
20
15
10
5
0
O_2
H_2
A–Cu^{2+} added
3.0
2.5
2.0
1.5
1.0
0.5
0.0
O_2 evolution μmol. ml^{-1}
300
200
100
0
$[O_2](\mu M)$
0
200
400
600
800
Time (min)

Fig. 32A

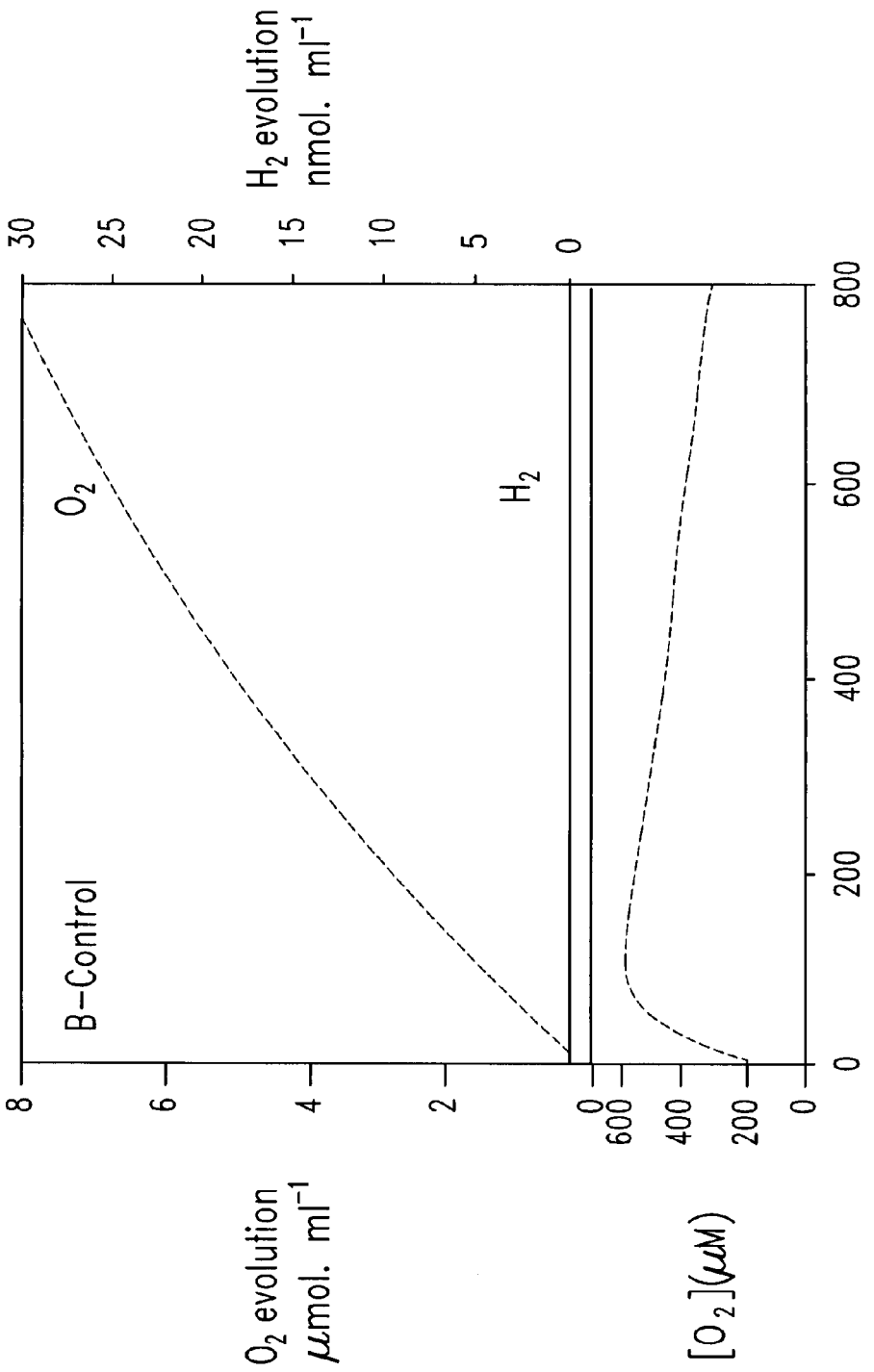

B-Control
O_2
H_2
O_2 evolution μmol. ml^{-1}
H_2 evolution nmol. ml^{-1}
$[O_2](\mu M)$
Time (min)
0
2
4
6
8
0
5
10
15
20
25
30
0
200
400
600
0
200
400
600
800

Fig. 32B

SYSTEM, METHOD, AND DEVICE FOR THE EXPRESSION OR REPRESSION OF PROTEINS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a U.S. national application under 37 C.F.R. §371(b) of International Application Serial No. PCT/US2007/017774 filed Aug. 11, 2007, which claims priority under 35 U.S.C §119(e) to U.S. Provisional Application Ser. No. 60/837,001, filed on Aug. 11, 2006, incorporated herein by reference.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on Aug. 12, 2011, is named 12377238SequenceListingCRF.txt and is 16,191 bytes in size.

FIELD OF THE INVENTION

This invention relates to systems, methods, and devices for inducing and/or repressing the expression of proteins. More particularly, the invention relates to systems, methods, and devices for inducing and/or repressing the expression of proteins in plastids.

BACKGROUND AND SUMMARY OF THE INVENTION

Proteins (e.g., peptides, oligopeptides, and polypeptides) are responsible for most of the activities of a cell, such as catalysis, communication, defense, movement, and transport. The underlying basis of a protein's biological activity is its amino acid sequence and/or its conformation. Accordingly, the biologically active portion of a protein should remain essentially intact and in its biologically functional conformation. The advancements in genetic engineering techniques for protein expression have led to the development of methods for the controlled expression of both native and foreign proteins in various systems in a form that maintains the biological activity of the proteins. Such genetically engineered, controlled expression systems can result in higher protein yield due to the expression of properly-folded, stable proteins where inactivation and degradation of the proteins is reduced as a result of the ability to control protein expression.

Although a number of different types of expression systems have been developed, in various organelles of host cells such as microorganisms, eukaryotic cells, including fungi, yeast, and mammalian cells, insect cells, etc., an expression system for the controlled expression of proteins utilizing stability factors of nuclear origin to regulate the expression of proteins in plastids has not previously been developed. Plastids are organelles responsible for photosynthesis and are commonly classified as chloroplasts, leucoplasts, amyloplasts, or chromoplasts. Plastids can differentiate or redifferentiate between these forms.

In one embodiment, a method for preparing an expression system for inducing the production of a protein in the plastid of a cell is provided. The method comprises the steps of introducing a first nucleic acid into the nucleus of a cell wherein the first nucleic acid encodes an inducible promoter, operatively linking the first nucleic acid to a second nucleic acid to form a recombinant nucleic acid wherein the second nucleic acid encodes a stability factor, wherein the introduction of an inducer or the removal of a repressor induces the expression of the stability factor, wherein the expressed stability factor associates in the plastid with an untranslated region of an mRNA stabilized by the stability factor and transcribed from a third nucleic acid, wherein the third nucleic acid is either native to the plastid or is foreign to the plastid and wherein the third nucleic acid encodes the protein, and wherein expression of the mRNA results in the production of the protein.

In another illustrative embodiment, a method for preparing an expression system for repressing the expression of a plastid protein in the plastid of a cell is provided. The method comprises the steps of introducing a first nucleic acid into the nucleus of a cell wherein the first nucleic acid encodes a repressible promoter, operatively linking the first nucleic acid to a second nucleic acid to form a recombinant nucleic acid wherein the second nucleic acid encodes a stability factor, wherein the introduction of a repressor or the removal of an inducer represses the expression of the stability factor, and wherein the repression of the expression of the stability factor results in the repression of expression of an mRNA stabilized by the stability factor and transcribed from a third nucleic acid, wherein the third nucleic acid is either native to the plastid or is foreign to the plastid and wherein the third nucleic acid encodes the protein, and wherein the expression of the protein is repressed.

In still another illustrative aspect, a method for expressing a plastid protein in the plastid of a cell is provided. The method comprises the steps of contacting the cell with an inducer or treating the cell under conditions that result in the removal of a repressor, wherein the inducer or the repressor associates with a first nucleic acid in the nucleus, wherein the first nucleic acid encodes an inducible promoter, wherein the first nucleic acid is operatively linked to a second nucleic acid to form a recombinant nucleic acid and wherein the second nucleic acid encodes a stability factor, expressing the stability factor, introducing the stability factor into the plastid wherein the stability factor associates in the plastid with an untranslated region of an mRNA to stabilize the mRNA wherein the mRNA is transcribed from a third nucleic acid which is either native to the plastid or is foreign to the plastid and wherein the third nucleic acid encodes the protein, expressing the mRNA, and producing the protein in the plastid.

In another embodiment, a method for repressing the expression of a plastid protein in the plastid of a cell is provided. The method comprises the steps of contacting the cell with a repressor or treating the cell under conditions that result in the removal of an inducer, wherein the repressor or the inducer associates with a first nucleic acid in the nucleus of the cell, wherein the first nucleic acid encodes a repressible promoter, wherein the first nucleic acid is operatively linked to a second nucleic acid to form a recombinant nucleic acid, and wherein the second nucleic acid encodes a stability factor, repressing the expression of the stability factor wherein the stability factor associates in the plastid with an untranslated region of an mRNA to stabilize the mRNA wherein the mRNA is transcribed from a third nucleic acid which is either native to the plastid or is foreign to the plastid and wherein the third nucleic acid encodes the protein, repressing the expression of an mRNA, and repressing the expression of the protein.

In still another embodiment, a system for expressing a plastid protein in the plastid of a recombinant host cell is provided. The system comprises an exogenously added inducer that induces the expression of a nuclear protein, the

recombinant host cell wherein the nucleus of the recombinant host cell comprises a recombinant nucleic acid, wherein the recombinant nucleic acid comprises a first nucleic acid operatively linked to a second nucleic acid to form the recombinant nucleic acid, wherein the first nucleic acid encodes an inducible promoter and wherein the second nucleic acid encodes a stability factor, and the plastid comprising a third nucleic acid that is either native to the plastid or foreign to the plastid wherein the third nucleic acid encodes the expressed plastid protein wherein the expression of the mRNA encoding the plastid protein is controlled by the stability factor.

In another illustrative embodiment, a system for repressing the expression of a plastid protein in the plastid of a recombinant host cell is provided. The system comprises an exogenously added repressor that represses the expression of a nuclear protein, the recombinant host cell wherein the nucleus of the recombinant host cell comprises a recombinant nucleic acid, wherein the recombinant nucleic acid comprises a first nucleic acid operatively linked to a second nucleic acid to form the recombinant nucleic acid, wherein the first nucleic acid encodes a repressible promoter and wherein the second nucleic acid encodes a stability factor, and the plastid comprising a third nucleic acid that is either native to the plastid or foreign to the plastid wherein the third nucleic acid encodes the expressed plastid protein and wherein the expression of the mRNA encoding the protein is controlled by the stability factor.

In another embodiment, a method for stimulating the production of hydrogen gas by expressing a plastid protein in the plastid of a cell is provided. The method comprises the steps of contacting the cell with an inducer or treating the cell under conditions that result in the removal of a repressor, wherein the inducer or the repressor associates with a first nucleic acid in the nucleus, wherein the first nucleic acid encodes an inducible promoter, wherein the first nucleic acid is operatively linked to a second nucleic acid to form a recombinant nucleic acid and wherein the second nucleic acid encodes a stability factor, expressing the stability factor, introducing the stability factor into the plastid wherein the stability factor associates in the plastid with an untranslated region of an mRNA to stabilize the mRNA wherein the mRNA is transcribed from a third nucleic acid which is either native to the plastid or is foreign to the plastid wherein the third nucleic acid encodes the protein, expressing the mRNA, producing the protein in the plastid, and producing hydrogen gas.

In yet another embodiment, a method for inhibiting the production of hydrogen gas by repressing the expression of a plastid protein in the plastid of a cell is provided. The method comprises the steps of contacting the cell with a repressor or treating the cell under conditions that result in the removal of an inducer, wherein the repressor or the inducer associates with a first nucleic acid in the nucleus of the cell, wherein the first nucleic acid encodes a repressible promoter, wherein the first nucleic acid is operatively linked to a second nucleic acid to form a recombinant nucleic acid, wherein the second nucleic acid encodes a stability factor, repressing the expression of the stability factor wherein the stability factor associates in the plastid with an untranslated region of an mRNA to stabilize the mRNA wherein the mRNA is transcribed from a third nucleic acid which is either native to the plastid or is foreign to the plastid and wherein the third nucleic acid encodes the protein, repressing the expression of the mRNA, repressing the expression of the protein, and inhibiting the production of hydrogen gas.

In still another embodiment, a method for stimulating the production of hydrogen gas by inducing and repressing the expression of a plastid protein in the plastid of a cell is provided. The method comprises the steps of sequentially i) contacting the cell with an inducer or treating the cell under conditions that result in the removal of a repressor and ii) contacting the cell with the repressor or treating the cell under conditions that result in the removal of the inducer, wherein the inducer or the repressor associates with a first nucleic acid in the nucleus, wherein the first nucleic acid encodes an inducible promoter, wherein the first nucleic acid is operatively linked to a second nucleic acid to form a recombinant nucleic acid and wherein the second nucleic acid encodes a stability factor, sequentially expressing and repressing the expression of the stability factor, wherein the stability factor associates in the plastid with an untranslated region of an mRNA to stabilize the mRNA wherein the mRNA is transcribed from a third nucleic acid which is either native to the plastid or is foreign to the plastid wherein the third nucleic acid encodes the protein, sequentially expressing and repressing the expression of the mRNA, producing the protein in the plastid, and producing hydrogen gas.

In any of the above-described embodiments, the first nucleic acid can be operatively linked to the second nucleic acid to form the recombinant nucleic acid prior to introducing the recombinant nucleic acid into the nucleus, the cell can have an inoperative copy or can be missing a copy or a homolog of the second or the third nucleic acid, the cell can be a plant cell or an algal cell, the plastid can be selected from the group consisting of a chloroplast, a leucoplast, a amyloplast, an etioplast, an elaioplast, and a chromoplast, the inducible promoter can have at least 90% sequence similarity to the Cyc6 promoter, and the third nucleic acid can encode a gene that has at least 90% sequence similarity to the psbD gene.

In any of the above-described embodiments, the inducer or repressor can be a chemical or an environmental condition where the chemical can be copper and where the environmental condition can be reduction in the concentration of oxygen to a predetermined level, the inducer can be applied and removed for a plurality of cycles wherein a cycle comprises applying and removing the inducer, the protein can be a protein involved in photosynthesis or in the production of hydrogen gas, the protein can be selected from the group consisting of a pharmaceutical agent, an industrial enzyme, an enzyme involved in chloroplast maturation or degradation, and a nutraceutical where the pharmaceutical agent is selected from the group consisting of an antibody, a vaccine antigen, and an antimicrobial agent, or other defense products for the host cell and the stability factor can be selected from the group consisting of Nac2 and Mbb1. In another illustrative embodiment, the second nucleic acid can code for a translational activating factor, such as, for example, Tbc2 or Tca1.

In another embodiment of the invention, a system and method for regulating the expression or repression of native or foreign genes in plastids is provided. In one embodiment, the invention relates to an expression system employing a nuclear-encoded chloroplast transcription factor, Nac2, the expression of which is regulated by an inducible promoter of the Cyc6 gene. In another embodiment, induction of Nac2 expression by an inducer (i.e., an agent or alteration of an environmental condition), such as low levels of oxygen, causes the expression of the psbD gene in the chloroplast. In yet another embodiment, an agent or environmental condition, such as removal of copper, that causes induction of the Cyc6 promoter, also causes expression of the psbD gene. In another illustrative embodiment, repression of the Nac2 gene by a repressor (i.e., an agent or alteration of an environmental condition), such as high levels of oxygen, results in no or reduced expression of the psbD gene. In a related embodi-

ment, an agent or alteration in an environmental condition that represses the inducible Cyc6 promoter also causes reduced expression of the psbD gene.

In other illustrative embodiments, the invention relates to the inducible expression of or the repression of a foreign gene in the chloroplast whereby replacement of the psbD gene in the chloroplast with a foreign gene facilitates inducible expression of or the repression of the foreign gene in the chloroplast by regulation of Nac2 expression.

In another embodiment, the invention relates to a method of producing hydrogen gas in the chloroplast through regulation of the psbD gene by Nac2 expression or repression of expression of Nac2. In this illustrative aspect of the invention, environmental conditions that facilitate the induction and repression of the Nac2 gene (e.g., reducing the level of oxygen to induce expression and elevating the level of oxygen to repress expression), resulting in the oscillating induction and repression of psbD gene expression, result in a reduction in the rate of photosynthesis and a resulting reduction in the concentration of oxygen. In this embodiment, the reduction in the concentration of oxygen facilitates the production of hydrogen. Thus, in one embodiment, the invention relates to a method of producing hydrogen gas by regulating the oscillating induction and repression of Nac2 and psbD gene expression.

In yet another embodiment, the invention relates to a method for enhancing the hydrogen-generating system through the recombinant expression of other genes in the chloroplast, for example, hydrogenases and repression of other recombinant or native proteins, such as phosphoribulose kinase.

In still another embodiment an apparatus for the production of hydrogen is provided. The apparatus comprises a first vessel configured to hold a cell culture in a substantially oxygen-depleted environment, a first pump in fluid communication with the first vessel and configured to pump a medium into the first vessel at a predetermined rate, and a measuring device coupled to the first vessel and configured to measure an amount of hydrogen produced by the cell culture.

In this embodiment, the first pump can be configured to pump the amount of medium into the first vessel at a rate substantially equal to the rate of growth of the cell culture, the first pump can comprise a peristaltic pump, the cell culture can comprise a cy6Nac2.49 culture, the measuring device can comprise a mass spectrometer, the apparatus can further comprise an agitation device coupled to the first vessel and operable to agitate the cell culture, the agitation device can comprise a magnetic stir bar, the apparatus can further comprise a second vessel configured to hold an amount of the medium, wherein the first pump is fluidly coupled to the second vessel and configured to pump the medium from the second vessel at the predetermined rate, the apparatus can further comprise a third vessel in fluid communication with the first vessel and configured to hold an overflow of the medium from the first vessel, and the apparatus can further comprise a filter and a second pump in fluid communication with the third vessel and the second vessel, the second pump being configured to pump an amount of the medium from the third vessel, through the filter, and into the second vessel.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention may be more readily understood by reference to the following figures wherein:

FIG. **1** shows a diagram of hydrogen and electron flow in the chloroplast of *C. reinhardtii* under aerobic and anaerobic conditions (from Kruse et al., 2005).

FIG. **2** shows a schematic outline of a plastid gene regulation system in which the nuclear inducible promoter is induced in the presence of an inducer. The left-hatched box represents the nuclear inducible promoter and the right-hatched box represents the gene for the stability factor. The filled box represents the stability factor association element, in this embodiment located in the 5' untranslated region of the plastid mRNA. The open box represents an mRNA produced from a plastid native gene. The cross-hatched box represents an mRNA produced from a foreign gene in the plastid.

FIG. **3** shows a schematic outline of a plastid gene regulation system in which the nuclear inducible promoter is repressed in the absence of an inducer. The left-hatched box represents the nuclear inducible promoter and the right-hatched box represents the gene for the stability factor. The filled box represents the stability factor association element, in this embodiment located in the 5' untranslated region of the plastid mRNA. The open box represents an mRNA produced from a plastid native gene. The cross-hatched box represents an mRNA produced from a foreign gene in the plastid.

FIG. **4** shows a schematic outline of a plastid gene regulation system in which the nuclear repressible promoter is repressed in the presence of a repressor. The left-hatched box represents the nuclear repressible promoter and the right-hatched box represents the gene for the stability factor. The filled box represents the stability factor association element, in this embodiment located in the 5' untranslated region of the plastid mRNA. The open box represents an mRNA produced from a plastid native gene. The cross-hatched box represents an mRNA produced from a foreign gene in the plastid.

FIG. **5** shows a schematic outline of a plastid gene regulation system in which the nuclear repressible promoter is induced in the absence a repressor. The left-hatched box represents the nuclear repressible promoter and the right-hatched box represents the gene for the stability factor. The filled box represents the stability factor association element, in this embodiment located in the 5' untranslated region of the plastid mRNA. The open box represents an mRNA produced from a plastid native gene. The cross-hatched box represents an mRNA produced from a foreign gene in the plastid.

FIG. **6** shows a schematic of the *Chlamydomonas* chloroplast genome with the psbD gene location shown. The arrow indicates the site of insertion of pSK108.

FIG. **7** shows a flow diagram for an apparatus for producing hydrogen.

FIG. **8** shows an apparatus for producing hydrogen.

FIG. **9** shows a schematic of the nuclear expression vector pSL17 used to transform nuclear Nac2 mutants and to introduce the Cyc6 promoter and the Nac2 gene. The map shows the arrangement of promoters, enhancer element from HSP70A promoter, and restriction sites for inserting the Cyc6 promoter and Nac2 gene. FIG. **9** discloses the "BstXI" sequence as SEQ ID NO: 9.

FIG. **10** shows a schematic of cy6Nac2(paroR).

FIGS. **11***a-c* show the genomic sequence of the Nac2 midi gene. (SEQ ID NO:10). The initiation codon is the first underlined ATG. The putative transit peptide is also underlined.

FIG. **12** shows the Cyc6 genomic sequence (SEQ ID NO: 11). The genomic sequences used to generate the fusion construct with the Nac2 midi gene are underlined. Also indicated (double-underlining) is the three base-pair difference in the cyc6Nac2 construct that created an NdeI restriction site.

FIG. **13** shows the growth properties of the cy6Nac2.49 transgenic strain.

FIG. **14** shows Western blot analysis of the cy6Nac2.49 transgenic strain.

FIG. **15** shows the hydrogen production of the cy6Nac2.49 transgenic strain.

FIG. **16** shows a map of the pSK108 vector. The pSK108 vector has flanking chloroplast DNA to direct it to the region surrounding the psbD gene.

FIG. **17** shows a map of pcg12.

FIG. **18** shows a map of the chloroplast expression plasmid pcg12 IBDV Flag.

FIG. **19** shows a map of the chloroplast expression plasmid pcg12 VP28 Flag.

FIG. **20** shows the chloroplast DNA sequence of psbD. (SEQ ID NO: 12). The underlined sequences were used to drive the expression of genes in the chloroplast using the cyc6Nac2 system.

FIG. **21** shows isolation of IND_aadA_X transgenic strains and growth on various media.

FIG. **22** shows Northern blot analysis of total RNA extracted from wild-type and isolation of IND_aadA_117.

FIG. **23** shows analysis of total and soluble (α-Nac 2) extracted from wild type and IND_aadA_117.

FIG. **24** shows screening for transgenic strains that induced the expression of three foreign proteins (DILP, IBVD, and VP28).

FIG. **25** shows inducible production of a foreign protein (DILP) using the Nac2 inducible chloroplast gene expression system.

FIG. **26** shows accumulation of psbD RNA, D2 and Nac2 proteins in cy6Nac2.49.

FIG. **27** shows restoration of constitutive PSII accumulation in cy6Nac2.49 by replacement of the psbD 5' UTR with the petA 5' UTR.

FIG. **28** shows inducible expression of the chloroplast psbD gene.

FIG. **29** shows a time course of copper-mediated repression of PSII synthesis in cy6Nac2.49.

FIG. **30** shows a time course of accumulation of PSII in cy6Nac2.49.

FIG. **31** shows expression of the psbD-aadA gene is induced by copper depletion and repressed by copper in the IND_aadA_117 transformants.

FIG. **32** shows hydrogen production in the cy6Nac2-49 strain.

DETAILED DESCRIPTION OF THE ILLUSTRATIVE EMBODIMENTS

As used herein, the phrase "foreign gene" or "foreign nucleic acid" (i.e., a transgene) means any nucleic acid inserted into a nucleic acid of a cell using recombinant DNA technology where the foreign gene or foreign nucleic acid is not normally present in that location in the cell. A foreign gene or foreign nucleic acid can include coding and noncoding nucleic acid sequences. A foreign gene or foreign nucleic acid can comprise a native nucleic acid that has been modified using recombinant DNA technology and has been reintroduced into the cell, or can comprise a native nucleic acid moved from one location to another within the cell.

As used herein, the phrase "native nucleic acid" or "native gene" means a nucleic acid that has its natural sequence (including naturally occurring mutations) and location in a cell.

As used herein, the term "inducible" means a promoter capable of being regulated so that mRNA transcripts are produced. Methods for determining the level of mRNA transcripts include Northern blotting and real-time PCR.

As used herein, the term "expression" can mean transcription of DNA into RNA or translation of RNA into protein.

As used herein the phrase "stability factor" means a nuclear protein that can exhibit activities, including, but not limited to, transcriptional, post-transcriptional, translational, post-translational, protein targeting, and protein recruitment activities, to enhance the expression or activity of a chloroplast protein.

The invention relates to systems, methods, and devices for the regulation of expression of a gene in the plastid of a cell (e.g., an algal or plant cell) for the purpose of producing useful products. The induction or repression of gene expression in the plastid is accomplished by transforming the nuclear genome of the cell with an inducible or repressible promoter operatively linked to a gene which codes for a chloroplast-targeted protein. The chloroplast-targeted protein associates, directly or indirectly (e.g., through accessory proteins), with an untranslated region of a plastid-expressed mRNA. The chloroplast-targeted protein is required for stability and/or translation of the plastid-expressed mRNA and hence expression of the plastid gene.

In one embodiment, the nuclear promoter is an inducible promoter and the addition or removal of a chemical (e.g., copper, a carbohydrate, or a protein) compound or an alteration in an environmental condition (e.g., low oxygen concentration, or an alteration in light, temperature, or nutritional status) activates the promoter resulting in expression of the chloroplast mRNA, and subsequently, the expression of the protein coded by the mRNA. In another embodiment, the nuclear promoter is a repressible promoter and the addition or removal of a chemical compound (e.g., copper, a carbohydrate, or a protein) or an alteration in an environmental condition (e.g., high oxygen concentration, or an alteration in light, temperature, or nutritional status) represses the promoter resulting in lack of expression of stability factor and inhibition of expression of the chloroplast mRNA, and subsequently, the expression of the protein coded by the mRNA.

In one embodiment, the invention relates to a method for expressing a protein in the plastid of a cell. The method comprises the steps of contacting the cell with an inducer or treating the cell under conditions that result in the removal of a repressor, wherein the inducer or the repressor associates with a first nucleic acid in the nucleus, wherein the first nucleic acid encodes an inducible promoter, wherein the first nucleic acid is operatively linked to a second nucleic acid to form a recombinant nucleic acid and wherein the second nucleic acid encodes a stability factor, expressing the stability factor, introducing the stability factor into the plastid wherein the stability factor associates in the plastid with an untranslated region of an mRNA to stabilize the mRNA wherein the mRNA is transcribed from a third nucleic acid which is either native to the plastid or is foreign to the plastid and wherein the third nucleic acid encodes the protein, expressing the mRNA, and producing the protein in the plastid.

In another embodiment, a method for repressing the expression of a plastid protein in the plastid of a cell is provided. The method comprises the steps of contacting the cell with a repressor or treating the cell under conditions that result in the removal of an inducer, wherein the repressor or the inducer associates with a first nucleic acid in the nucleus of the cell, wherein the first nucleic acid encodes a repressible promoter, wherein the first nucleic acid is operatively linked to a second nucleic acid to form a recombinant nucleic acid, and wherein the second nucleic acid encodes a stability factor, repressing the expression of the stability factor wherein the stability factor associates in the plastid with an untranslated region of an mRNA to stabilize the mRNA wherein the mRNA is transcribed from a third nucleic acid which is either native to the plastid or is foreign to the plastid and wherein the

third nucleic acid encodes the protein, repressing the expression of an mRNA, and repressing the expression of the protein.

In the above-described embodiments, the protein is expressed in the plastid of a cell. Illustratively, the cells can be cells of plants or algal cells or any cell type that contains a plastid. Plastids are organelles that contain a plastid genome, often in multiple copies. Plastids are found in, for example, plants and algae and include chloroplasts, leucoplasts, amyloplasts, etioplasts, elaioplasts, and chromoplasts.

In the method and system embodiments described herein, the third nucleic acid encodes the protein of interest. In one illustrative embodiment, the third nucleic acid, coding for the expressed protein, can be either native to the plastid or foreign to the plastid (i.e., a transgene). In this embodiment, the expressed protein (e.g., a peptide, an oligopeptide, or a polypeptide) can be expressed under the control of an inducible or repressible promoter and includes proteins involved in photosynthesis, such as components of Photosystem I or II (e.g., psbA and psbD and the D1 and D2 subunits of Photosystem II), proteins involved in CO_2 fixation (e.g., phosphoribulose kinase), hydrogenases (e.g., HydA1 and HydA2), and proteins that regulate the activity of any of these proteins (e.g., the assembly of any these proteins (e.g., HydEF and HydG)), or any other proteins that are native to the plastid. Exemplary native proteins that can be expressed under the control of an inducible or repressible promoter include any of the proteins involved in the regulation of the photosynthetic processes or carbon assimilation processes depicted or implied in FIG. **1** or any other protein native to the plastid. In an alternate embodiment, an amino acid, such as an aromatic amino acid, or an amino acid precursor can be produced by regulating the expression of proteins native to the plastid that are involved in the synthetic pathways for amino acids, such as aromatic amino acids.

In another illustrative embodiment, a protein involved in the production of hydrogen gas can be expressed under the control of an inducible or repressible promoter, or both, using the methods, systems, and devices described herein. In this embodiment, proteins involved in the production of hydrogen gas can be any proteins described in this or the preceding paragraph, or can be any of the proteins which are involved in the regulation of the photosynthetic processes or carbon assimilation processes depicted or implied in FIG. **1**. In this embodiment, the inducer or repressor can be applied and removed for a plurality of cycles, wherein a cycle comprises applying and removing the inducer or repressor.

In another embodiment, the third nucleic acid, coding for the expressed protein, can be foreign to the plastid (i.e., a foreign nucleic acid or a foreign gene). In this embodiment, the protein can be a pharmaceutical agent, an industrial enzyme, an enzyme involved in chloroplast maturation or degradation, or a nutraceutical. In this embodiment, the expressed protein can be, for example, an antibody, a vaccine antigen (e.g., for use in a vaccine), an antimicrobial agent, or other defense products for the host cell, a growth hormone, a cytokine, such as an interleukin or an interferon, insulin, colony-stimulating factors, coagulation factors, erythropoietins, growth factors, such as epidermal growth factor, somatotropin, fibroblast growth factor, platelet-derived growth factor, and the like, amylases, proteases, lipases, pectinases, cellulases, hemicellulases, pentosanases, xylanases, and phytases, insecticidal proteins, phenyl ammonia lyase, or any other pharmaceutical agent, industrial enzyme, or nutraceutical that is proteinaceous.

In yet another illustrative embodiment, additional nucleic acids (e.g., a fourth nucleic acid, etc.) coding for an expressed protein, can be expressed in the chloroplast and can be native or foreign to the plastid (i.e., a foreign nucleic acid or a foreign gene). In these embodiments, the expression of the additional nucleic acids can be controlled by their own stability factors coded by additional nucleic acids in the nucleus (i.e., similar to the second nucleic acid) or the expression of these additional nucleic acids can be controlled by the stability factor encoded by the second nucleic acid. In one illustrative embodiment, one stability factor associates with the stability factor association element in plastid mRNA and stimulates expression of the third nucleic acid and additional nucleic acids (e.g., the fourth nucleic acid, etc.) operatively linked to the third nucleic acid. In these embodiments, the protein expressed can be a pharmaceutical agent, an industrial enzyme, an enzyme involved in chloroplast maturation or degradation, or a nutraceutical. In this embodiment, the expressed protein can be, for example, an antibody, a vaccine antigen (e.g., for use in a vaccine), an antimicrobial agent, or other defense products for the host cell, a growth hormone, a cytokine, such as an interleukin or an interferon, insulin, colony-stimulating factors, coagulation factors, erythropoietins, growth factors, such as epidermal growth factor, somatotropin, fibroblast growth factor, platelet-derived growth factor, and the like, amylases, proteases, lipases, pectinases, cellulases, hemicellulases, pentosanases, xylanases, and phytases, insecticidal proteins, phenyl ammonia lyase, or any other pharmaceutical agent, industrial enzyme, or nutraceutical that is proteinaceous.

In the embodiment where the expressed protein is a vaccine antigen for use as a vaccine, the expressed protein, or a portion thereof, can be located on or in an organelle of the cells, such as algal or plant cells. The algae, for example, can then be lysed and the vaccine antigen can be used for inducing an immune response in a host animal to a pathogen if the vaccine antigen is at least partially derived from a pathogenic organism.

In one embodiment, the algae with the vaccine antigen are administered as a food substance. Exemplary animals to which the vaccines can be administered include, but are not limited to, mammals, birds, and aquaculture species. In particular, the vaccine can be administered to aquatic vertebrates such as all vertebrate fish, which may be bony or cartilaginous fish, including, but not limited to, salmonids (including trout, salmon, and Artic char), carp, catfish, yellowtail, seabream, and seabass. Such a vaccine can also be administered to shellfish including, but are not limited to, clams, lobster, shrimp, crab, and oysters. Exemplary methods of delivery include oral administration, as a dried powder, as a component of the normal diet, and by immersion of the animal in a suspension containing the vaccine.

In the case of aquatic vertebrates, examples of pathogenic organisms whose antigenic determinants may be expressed as vaccine antigens on the surface of cells using the methods and systems described herein include, but are not limited to *Renibacterium salmoninarum* (causative agent of bacterial kidney disease in salmon, trout, char and whitefish; i.e., salmonids), *Aeromonas salmonicida, Aeromonas hydrophila*, species of *Vibrio* (including *V. anguillarum* and *V. ordalii*), species of *Pasteurella* (including *P. piscicida*), species of *Yersinia*, species of *Streptococcus, Edwardsiella tarda* and *Edwardsiella ictaluria*, the viruses causing viral hemorrhagic septicemia, infectious pancreatic necrosis, viremia of carp, infectious hematopoietic necrosis virus, channel catfish virus, grass carp hemorrhagic virus, nodaviridae such as nervous necrosis virus or striped jack nervous necrosis virus, infectious salmon anaemia virus, and the parasites *Ceratomyxa shasta, Ichthyophthirius multifillius, Cryptobia salmo-*

sitica, Lepeophtheirus salmonis, Tetrahymena species, *Trichodina* species and *Epistylus* species.

In the embodiment where the protein is expressed in algae, the algae can be, for example, green algae. For example, algae that can be used include Chlorophyta such as Charoides (e.g., Charoides, Lamprothamnium, Nitellopsis, and *Nitella*), *Zynematales* (e.g., *Zygnema, Closterium*, and *Netrium*), Codials (e.g., *Codium fragile, Helimida opunta*, and *Caulerpa*), *Bryopsis plumosa* (e.g., *Bryopsis, Pseudobryopsis, Bryopsidella, Derbesis*, and *Pedobesia*), *Acetabularia Ryukyuensis* (e.g., *Acetabularia Ryukyuensis, Halicoryne wrightii, Neomeris annulata, Cymopolia van bossei, Bornettella ovalis*, and *Acetabularia calyculus*), *Siphonocladales* (e.g., Valoniaceae and Boodleaceae), *Cladophora* (e.g., Anadyomene writii, Cladophora, Cladophora sauteri, and Chaetomorpha), *Ulva* (e.g., *Ulva* and Fnteromorpha), Ulotrichales (e.g., Acrosiphoniaceae, Collinsiellaceae, Monostromaceae, and Chlorocystidaceae), Prasiola, *Chlorella*, Chlorococcales (e.g., *Pediastrum* and *Hydrodictyon*), and Volvocales (e.g., *Chlamydomonus, Pandorina*, Pleodorina, and *Volvox*).

Exemplary algae that typically can be used in any of the embodiments described in this application include *Chlamydomonas* species, particularly *Chlamydomonas reinhardtii, Chlorella* species, and *Volvox* species. *Chlamydomonas reinhardtii*, a unicellular eukaryotic green algae is particularly advantageous. *Chlamydomonas* strains are available, for example, from *Chlamydomonas* Genetic Stock Center, Duke University (Durham, N.C.). Auxotrophic mutants of *Chlamydomonas reinhardtii* (mutants that differ from the wild-type in requiring one or more nutritional supplements for growth) are readily available at the *Chlamydomonas* Genetic Stock Center and such mutants can be genetically complemented by the transforming DNA exogenous DNA introduced into the cell), which facilitates selection of algae containing a desired transgene. In other embodiments disabled algae can be used. Disabled algae are genetically engineered such that they will not proliferate unless they are in very specific controlled environments (i.e., such strains will not grow or transfer their genes in the wild). Within the context of this disclosure, such algae are said to be "disabled." Use of such disabled strains inhibits or limits spread of the transgenic algae used in the present invention into the environment.

Exemplary plants suitable for use in the methods and systems described herein include cultured plant cells (protoplasts and callus cells) and whole plants (single cell and multicellular plants). In various embodiments, the plant cells (i.e., cultured plant cells or cells of whole plants) can be from plants including oat, wheat, rye, barley, rice, safflower, maize, legumes, such as alfalfa, soy bean, tomato, sugar beet, and potato plants. Other useful plants can be, for example, fruit-bearing plants, such as plants that bear apples, pears, cherries, grapes, citrus fruits, pineapples and bananas, and trees, such as larch. Other suitable plants include oil palms, tea, cocoa and coffee shrubs, tobacco, cotton, flax, sunflower, pasture grasses, forage cereals, feed plants, and peanut and lentil plants. Other useful plants include Arabidosis, soapworts (*Saponaria*), duckweed (Leminacea), ferns, mosses, and liverworts. In this embodiment, vectors commonly used in genetic engineering in plants can be used for the transfer of the nucleic acid molecules according to the invention to plant cells.

In the methods and systems described herein the first and second nucleic acids are introduced into the cells (e.g., algal or plant cells). In this embodiment, the first nucleic acid encodes a promoter that is either inducible, repressible, or both inducible and repressible, and the second nucleic acid encodes a stability factor (e.g., Nac2 or Mbb1) that regulates the expression of a plastid mRNA. In another illustrative embodiment, the second nucleic acid can code for a translational activating factor, such as, for example, Tbc2 or Tca1.

The inducible or repressible promoter controls the expression of the stability factor. In various embodiments, any suitable type of inducer or repressor (e.g., a chemical or modified environmental condition) can be used depending on the nuclear promoter being used. An exemplary promoter suitable for use in the methods and systems described herein is the Cyc6 promoter (see FIG. **12**). Any other suitable promoters can be used including promoters with sequence similarity to the Cyc6 promoter sequence, such as 60%, 70%, 80%, 85%, 90%, 95%, or 98% sequence similarity to the Cyc6 promoter. Also, sequences capable of hybridizing to the complement of the Cyc6 promoter under stringent hybridization conditions can be used. Other suitable inducible or repressible promoters that can be used include promoters that respond to factors such as environmental conditions (such as anoxia, heat, drought, or light), chemicals, nutrients, hormones, pathogens, injury, herbivory, developmental stage, and tissue type. Such promoters are known to those skilled in the art. Promoters may respond to more than one factor, and a single factor may activate or repress more than one promoter.

In addition to Cyc6, other inducible promoters in *Chlamydomonas* include the promoter for the CO_2-induced plasma-membrane protein gene (Genbank accession no. U31976), the promoter for the FEA1 gene which is tightly controlled by iron availability (Sasaki et al., 1998; Rubinelli et al., 2002), and the promoter of the Nit1 gene that is negatively repressed in the presence of ammonium and glutamate and induced in media lacking ammonium (Fernández 1989). Examples of inducible promoters in higher plants include the light inducible promoter of the small subunit of Rubisco, the U-V inducible promoter of the chalcone synthase gene, the coumaric-acid inducible promoter of the chalcone synthase gene, the hypoxia inducible promoter of the alcohol dehydrogenase gene, and the pathogenesis-induced promoters (PR-1-14) of tobacco, tomato, cucumber, and *arabadopsis.*

The stability factor is expressed under the control of the inducible and/or repressible promoter and is introduced into the plastid where the stability factor associates, directly or indirectly (e.g., through accessory proteins), in the plastid with an untranslated region of an mRNA to stabilize the mRNA. In various embodiments, the untranslated region of the mRNA can be at the 5' or the 3' end of the mRNA. The mRNA is transcribed from the third nucleic acid which encodes the expressed protein. In another illustrative embodiment, mRNA can be transcribed from additional nucleic acids operatively linked to the third nucleic acid or not linked to the third nucleic acid and controlled by their own stability factors. Thus, the expressed protein is produced in the plastid under the control of the stability factor, the expression of which is controlled by the inducible and/or repressible nuclear promoter.

In one illustrative aspect, the stability factor can associate, directly or indirectly, not only with untranslated regions of the mRNA either at the 5' or the 3' end of the mRNA, or both, but the stability factor can also associate, directly or indirectly, with coding regions of the mRNA. In another illustrative aspect, the stability factor can associate directly or indirectly with the mRNA, for example, by association with other accessory proteins in a complex where the other proteins associate directly or indirectly with the mRNA untranslated and/or coding regions.

In one embodiment, the first and second nucleic acids are incorporated into nuclear DNA employing, for example, inte-

gration or recombination (e.g., homologous recombination or other types of recombination). In another embodiment, the first and second nucleic acids are expressed using an expression vector that is introduced into the cells. In this embodiment, the first and second nucleic acid inserts in the vector do not recombine with nuclear DNA, but, rather, the second nucleic acid, coding for the stability factor, is expressed autonomously using regulatory sequences present in the vector including the inducible and/or repressible promoter coded by the first nucleic acid. Any suitable vector known to those skilled in the art can be used including the vectors described herein in Examples 1-4.

In each of these embodiments, the first nucleic acid is operatively linked to the second nucleic acid to form a recombinant nucleic acid. An exemplary recombinant nucleic acid described herein is the Cyc6 promoter (i.e., the first nucleic acid) operatively linked to the Nac2 coding sequence (i.e., the second nucleic acid). The first and second nucleic acids can be operatively linked to each other by using cloning methods well-known to those skilled in the art, including methods of digesting nucleic acids with restriction enzymes and ligating the first and second nucleic acids to one another and to the ends of a digested vector, using ligases. Such cloning methods are described, for example, in Sambrook et al., "Molecular Cloning: A Laboratory Manual", 3rd Edition, Cold Spring Harbor Laboratory Press, (2001), incorporated herein by reference, or in S. Surzycki, "Basic Techniques in Molecular Biology," Springer-Verlag (2000), incorporated herein by reference.

In embodiments where the second nucleic acid is expressed autonomously under the control of the promoter coded for by the first nucleic acid, the expression construct (i.e., the vector-insert construct) typically comprises a transcription terminator for terminating the transcription of the coding sequence present in the second nucleic acid, and can contain other 5' and 3' regulatory sequences. The transcription terminator is typically present in the second nucleic acid, but can be incorporated into the vector.

In embodiments where the first and second nucleic acids are stably incorporated into nuclear DNA, the transcription terminator is typically present in the second nucleic acid, but can be part of the nuclear DNA sequence. The additional 5' and 3' regulatory sequences can be present in the first or second nucleic acids, in the vector, and/or in nuclear DNA, such as transcriptional enhancer elements and sequences involved in mRNA stabilization. In this embodiment, the vector can also contain nuclear targeting sequences that facilitate integration of the recombinant nucleic acid into nuclear DNA. In one embodiment, the nucleus can have an inoperative copy or can be missing a copy or a homolog of the second nucleic acid.

In various embodiments described herein, the vectors for autonomous expression of the stability factor or for incorporation of the recombinant nucleic acid into nuclear DNA have a bacterial origin of replication for replicating the vector construct to make large-scale preparations of desired vectors with or without the inserted recombinant nucleic acid (i.e., the operatively linked first and second nucleic acids) for use in cloning. The vectors also typically have restriction endonuclease cleavage sites for the insertion of DNA fragments (e.g., a multiple cloning site), and selectable genetic markers for the selection of transformants. The selectable marker can be a marker, such as the aadA gene or nptII, which allows for growth of the transformed cells on media supplemented with antibiotics (Goldschmidt-Clermont, *Nucl. Acids Res.*, vol. 19, pages 4083-4089 (1991)). Both native gene (arg7, nit1) and foreign gene (ble, aphVIII, aadA, nptII) selectable markers have been developed as reporter genes for nuclear transformation.

The vectors with the recombinant nucleic acid (i.e., an insert comprising the first and second nucleic acids) are introduced into the cells (e.g., algal or plant cells) by standard transformation techniques well-known to those skilled in the art. Exemplary transformation methods include electroporation, glass-bead mediated DNA delivery, the use of polyethylene glycol-mediated transformation, biolistics, and the like.

In one embodiment, for transforming algae, autolysin, an enzyme which is released during mating and degrades cell walls is used to break down the cell wall before transformation. In an alternate embodiment, mutant strains which lack the ability to synthesis the cell wall (e.g., cw15cw10) have been generated and can be used for efficient transformation.

In one illustrative aspect, transformants can be detected by PCR and Southern blotting. Other procedures known to those skilled in the art can also be used, such as for example, antibiotic addition, copper addition, antibody detection (e.g., ELISA and Western blotting), and sequencing. The choice of such procedure depends upon the construct used.

If a transgene is to be expressed in the plastid, the third nucleic acid can be incorporated into a vector and the vector constructs can be made and can be replicated generally as discussed above for the first and second nucleic acids. Illustratively, plastid transformation can be achieved with biolistics, in which the vector containing the transgene is introduced into the cell on a gold or tungsten microparticle accelerated by an inert gas, such as helium. This method may cause less damage to the cells. In a related illustrative embodiment, the cells to be transformed can be plated on selective solid agar media and DNA-coated tungsten beads can be delivered into the plastid by accelerating them with helium gas or gun powder. Using this technique, efficient delivery of recombinant DNA into the plastid can be achieved.

In one embodiment, detection of integration of the third nucleic acid into plastid DNA can rely on expression of heterologous DNA encoding the bacterial aminoglycoside adenyl transferase gene (aadA). This embodiment enables a method of selecting transformants using the antibiotic spectinomycin or streptomycin. Using this reporter construct, it is possible to specifically insert, disrupt, modify, mutate or delete any non-essential gene or cis acting elements in the plastid.

In another illustrative embodiment, chloroplast transformation with a transgene can be achieved using a transgene flanked by homologous chloroplast targeting sequences that facilitate integration of the transgene into the chloroplast DNA. In one embodiment, integration of the transgene can occur by two homologous recombination events between the flanking chloroplast sequences of the vector and their homologous sequences in the chloroplast genome. In one embodiment, the chloroplast can have an inoperative copy or can be missing a copy or a homolog of the third nucleic acid.

Exemplary modifications of nucleic acids that make the nucleic acids foreign include, but are not limited to, modifying the nucleic acids to achieve codon optimization, ligating the nucleic acids to 5' or 3' untranslated regions of other genes, adding promoter or termination sequences to nucleic acids, ligating sequences useful for homologous recombination, and adding other elements required for gene expression, targeting, or stabilization, for example, to the nucleic acids. Additional modifications include fusing the nucleic acid to the nucleic acid of other native or transgenes to form a fusion protein.

Exemplary schematic outlines of plastid gene regulation systems that are within the scope of this invention are shown in FIGS. **2-5**. FIG. **1** shows a schematic outline of a plastid gene regulation system in which the nuclear inducible promoter is induced in the presence of an inducer. In this embodiment, the inducer (e.g., an environmental condition such as low oxygen levels) induces the promoter that regulates expression of the stability factor and the stability factor is expressed. Following translation, the stability factor is targeted to the plastid where it associates with a specific mRNA, depending on the stability factor being expressed, and the mRNA and the protein it encodes are expressed.

FIG. **3** shows a schematic outline of an exemplary plastid gene regulation system in which the nuclear inducible promoter is repressed in the absence of an inducer. In this embodiment, the absence of the inducer, the inducible promoter is not activated and the stability factor is not expressed. Without the stability factor, the mRNA in the plastid is degraded or not translated and the protein is not expressed.

FIG. **4** shows a schematic outline of an exemplary plastid gene regulation system in which the nuclear repressible promoter is repressed in the presence of a repressor (e.g., a predetermined level of copper in the medium). In this embodiment, in the presence of the repressor, the repressible promoter is not activated and the stability factor is not expressed. Without the stability factor, the mRNA in the plastid is degraded or not translated and the protein is not expressed.

FIG. **5** shows a schematic outline of an exemplary plastid gene regulation system in which the nuclear repressible promoter is induced in the absence a repressor (e.g., a reduced level of copper in the medium or the absence of copper in the medium). In this embodiment, in the absence of the repressor the nuclear promoter that regulates expression of the stability factor is induced and the stability factor is expressed. Following translation, the stability factor is targeted to the plastid where it associates with a specific mRNA, depending on the stability factor being expressed, and the mRNA and the protein it encodes are expressed.

In embodiments where it is desirable to isolate and purify the expressed proteins obtained using the methods and systems described herein, the proteins can be expressed and then purified using conventional techniques. For example, the proteins can be obtained in a form that is about 40% pure, about 50% pure, about 60% pure, about 70-80% pure, about 90% pure, about 95% pure, or about 98% pure. For purification from the cells, a lysate can, for example, be subjected to ammonium sulfate precipitation followed by DEAE-Sepharose column chromatography. Other conventional techniques known to those skilled in the art can be used such as gel filtration, ion exchange chromatography, DEAE-Sepharose column chromatography, affinity chromatography (such as using the FLAG-tagged system described in Example 2 below), solvent-solvent extraction, ultrafiltration, and HPLC. Alternatively, purification steps may not be required because the proteins may be present in such high concentrations that the protein is essentially pure in the lysate (e.g., 70-80% pure). The expressed protein can be concentrated by such techniques as, for example, ultrafiltration and tangential flow filtration.

In one embodiment, the cells can be lysed, for example, by sonication, heat, or chemical treatment, and the homogenate centrifuged to remove cell debris. The supernatant can then be subjected to ammonium sulfate precipitation, and additional fractionation techniques as required, such as gel filtration, ion exchange chromatography, DEAE-Sepharose column chromatography, affinity chromatography, solvent-solvent extraction, ultrafiltration, and HPLC to purify the expressed protein. It should be understood that the purification methods described above for purification of the expressed proteins from the culture medium or from cells are nonlimiting and any purification techniques known to those skilled in the art can be used to purify the expressed proteins if such techniques are required to obtain a substantially pure protein.

The cells (e.g., algal or plant cells) can be cultured using a variety of techniques to promote protein expression. Culture media for cells, including algal and plant cells, are known in the art and are typically supplemented with a carbon source (e.g., glucose or acetate). The cells can be cultured to maintain a desired density, for example, as described below using a culture system and device useful for the production of hydrogen gas as an example.

As discussed in detail above, the expression methods and systems described herein may be used to produce hydrogen. Thus, in another embodiment an apparatus for the production of hydrogen is provided. The apparatus comprises a first vessel configured to hold a cell culture in a substantially oxygen-depleted environment, a first pump in fluid communication with the first vessel and configured to pump a medium into the first vessel at a predetermined rate, and a measuring device coupled to the first vessel and configured to measure an amount of hydrogen produced by the cell culture.

FIG. **7** illustrates an apparatus **10** for producing hydrogen that may be used in some embodiments. The apparatus **10** includes a fresh medium storage vessel **12**, a pump **14**, a reaction system **16**, and an overflow vessel **18**. The fresh medium storage vessel **12** may be embodied as any type of vessel capable of storing an amount of medium, such as TAP (acetate) or HSM (minimal), in a substantially hermetically-sealed environment. In some embodiments, an amount of gas **24**, such as argon gas, is pumped into the fresh medium storage vessel **12** via a conduit **26** to purge the vessel **12** of oxygen to thereby form a substantially oxygen-depleted environment therein. The conduit **26** may be embodied as any type of tube, line, or other conduit capable of facilitating passage of a fluid (e.g., a gas) into the fresh medium storage vessel.

The pump **14** is fluidly coupled to the fresh medium storage vessel **12** via a conduit **28** and to the reaction system **16** via a conduit **30**. The conduit **28** (and the conduit **26** if included) is coupled to the vessel **12** such that the substantially hermetically-seal environment of the fresh medium storage vessel **12** is maintained. The conduits **28**, **30** may be embodied as any type of tubes, lines, or other conduits capable of facilitating passage of a fluid via a pumping action provided by the pump **14**. The pump **14** may be embodied as any type of pump capable of pumping an amount of medium from the fresh medium storage vessel **12** to the reaction system **16** at a predetermined rate without adversely interacting with the fresh medium. For example, in one particular embodiment, the pump **14** is embodied as a peristaltic pump such that the potential for damage to the medium during the pumping process is reduced.

The reaction system **16** includes a reaction vessel **20** and an agitation device **22**. The reaction vessel **20** is substantially similar to fresh medium storage vessel **12** and may be embodied as any type of vessel capable of storing an amount of medium and algae culture, or other type of host cell, in a substantially hermetically-sealed environment. Fresh medium is pumped from the fresh medium storage vessel **12** to the reaction vessel **16** via the conduits **28**, **30** and the pump **14**. Because the fresh medium is stored in a substantially oxygen-depleted environment, the likelihood of inadvertently introducing oxygen into the reaction vessel **16** is reduced. The conduit **30** is coupled to the reaction vessel **20**

such that the substantially hermetically-sealed environment of the reaction vessel **12** is maintained.

The agitation device **22** is operably coupled to the reaction vessel **20** and may be embodied as any type of device capable of maintaining the culture stored in the reaction vessel **20** in an agitated state. For example, the agitation device **22** may be embodied as an automated stirring device such as a magnetic stir bar assembly or the like.

A conduit **32** drains any overflow of culture from the reaction vessel **20** to an overflow vessel **18**. The overflow vessel **18** may be substantially similar to vessels **12**, **20** and may be embodied as any type of vessel capable of storing an amount of culture therein. The conduit **32** is substantially similar to conduits **28**, **30** and may be embodied as any type of tube, line, or other conduit capable of facilitating passage of a fluid from the reaction vessel **20** to the overflow vessel **18**. Similar to conduit **30**, the conduit **28** is coupled to the reaction vessel **20**, such the that substantially hermetically-sealed environment of the reaction vessel **20** is maintained. The conduit **30** may be coupled to the reaction vessel **20** in a position such that the level of culture contained in the reaction vessel **20** remains at (or within) a predetermined level(s) by expelling a portion of the culture into the overflow vessel **18**.

In some embodiments, a measuring device **34** may be coupled to the reaction vessel **20** via a communication link **36** such that the amount of hydrogen or other gas produced by the culture in the vessel **20** may be measured. The measuring device **34** may be embodied as any type of device capable of measuring an amount of the gas of interest. In one particular embodiment, the measuring device **34** is embodied as a mass spectrometer, but in other embodiments, other types of measuring devices may be used. The communication link **36** may be any type of communication link capable of facilitating the communication of data to the measuring device **24** such as, for examples, any number of wires, cables, fiber optic cables, tubes, conduits, or the like. In one particular embodiment, the communication link **36** includes an electrode portion positioned in the reaction vessel **20**. The electrode portion may be, for example, a silver electrode.

In some embodiments, the apparatus **10** may include a filter system **40** and a secondary pump **38**. The filter system **40** is coupled to the overflow vessel **18** via a conduit **42** and to the pump **38** via a conduit **44**. The pump **38** is coupled to the fresh medium storage vessel **12** via a conduit **46**. The conduits **42**, **44**, **46** are substantially similar to conduits **28**, **30**, **32** and may be embodied as any type of tube, line, or other conduit capable of facilitating passage of a fluid. The pump **38** may be similar to the pump **14** and may be embodied as any type of pump capable of pumping an amount of "spent" culture from the overflow storage vessel **18** to the reaction fresh medium storage vessel **12** at a predetermined rate without adversely interacting with the fresh medium. For example, in one particular embodiment, the pump **38** is embodied as a peristaltic pump. The filter system **40** may be embodied as any number and type of filters and associated interconnects that are capable of filtering the culture stored in the overflow vessel **18**.

In operation, the pump **14** is configured to pump fresh medium from the fresh medium storage vessel **12** to the reaction vessel **20** of the reaction system **16** at a predetermined rate. In one particular embodiment, the pump **14** is configured to pump the fresh medium into the reaction vessel **20** at a rate substantially equal to a rate of cell growth of an algae stored in the reaction vessel **20**. As discussed in detail above, because the algae, or other host cell type, is stored in the reaction vessel **20** in a substantially oxygen-depleted environment desirable gene expression is induced in the algae, or other host cell type, such that hydrogen production is increased. The substantially oxygen-depleted environment of the reaction vessel **20** is maintained while introducing fresh medium from the fresh medium storage vessel **12** because such fresh medium is also stored in a substantially oxygen-depleted environment in the vessel **12** as discussed above. Alternatively, in other embodiments, the algae, or other host cell type, stored in the reaction vessel **20** may be self-induced. Regardless, it should be appreciated that apparatus **10** is a single phase apparatus. That is, the algae, or another host cell type, stored in the reaction vessel **20** proliferate and are induced in the same vessel.

As fresh medium is introduced into the reaction vessel **20**, the agitation device **22** is configured to keep the culture stored in the reaction vessel in a continual state of agitation. In addition, as fresh medium is introduced, a portion of the existing culture is removed from the reaction vessel to the overflow vessel **18**. In this way, the amount of algae, or another host cell type, contained in the reaction vessel **20** is maintained at a substantially constant value. In embodiments including the pump **38** and the filter system **40**, the pump **38** removes an amount of "spent" culture from the overflow vessel and reintroduces such medium into the fresh medium storage vessel after being filtered by the filter system **40** to remove any algae, or other host cell type, contained therein.

Referring now to FIG. **8**, in one particular embodiment, an apparatus **50** for producing hydrogen includes a fresh medium storage vessel **52**, a peristaltic pump **54**, a reaction system **56**, and an overflow vessel **62**. The fresh medium storage vessel **52** is substantially similar to the fresh medium storage vessel **12** and is illustratively embodied as a 0.25 liter vessel. An amount of fresh medium **64**, illustratively embodied as TAP (acetate) or HSM (minimal), is stored in the vessel **52**. A cap **66** is coupled to the vessel **52** such that an inner cavity **68** of the vessel **52** is substantially hermetically-sealed from the outside environment. A conduit **70** is coupled to the cap **66** and includes an end portion **72** positioned in the inner cavity **68** of the vessel **52**. An amount of argon gas **74** is introduced into the inner cavity **68** via the conduit **70** to thereby substantially purge the inner cavity of oxygen.

The peristaltic pump **54** is coupled to the fresh medium storage vessel **52** via a conduit **76** and to the reaction system **56** via a conduit **78**. The peristaltic pump **54** is illustratively embodied as a model IP4 peristaltic pump, which is commercially available from Ismatec of Glattburg, Switzerland. The pump **54** is configured to pump an amount of fresh medium from the fresh medium storage vessel **52** to the reaction system **56** at a predetermined rate substantially equal to the growth rate of an algae culture, or another host cell type, stored in the reaction system **56**.

The reaction system **56** includes a reaction vessel **88**, illustratively embodied as a 0.25 liter vessel, and a magnetic stir bar system **90** configured to continually agitate an amount of medium and algae culture **94**, or another host cell type, stored in the reaction vessel **88**. The magnetic stir bar system **90** is illustratively embodied as a model KM02 magnetic stir bar, which is commercially available from Milian of Geneva, Switzerland. A cap **92** is coupled to the reaction vessel **88** such that an inner cavity **94** of the vessel **88** is substantially hermetically-sealed from the outside environment. The conduit **78** is coupled to the cap **92** and includes an end portion **96** positioned in the inner cavity **94** of the reaction vessel **88**.

A mass spectrometer **100** is also coupled to the reaction vessel **88** via a communication link **102**. The mass spectrometer **100** is illustratively embodied as a model MM8-80 mass spectrometer, which is commercially available from VG Instruments of Cheshire, United Kingdom. The communica-

tion link **102** includes an electrode **104** positioned in the inner cavity **94** of the vessel **88**. The mass spectrometer **100** is configured to measure the amount of hydrogen (and, in some embodiments, the amount of oxygen) produced by the medium and algal culture, or another host cell type, stored in the reaction vessel **88**.

Additionally, a conduit **106** is coupled to the cap **92** and includes an end portion **108** positioned in the inner cavity **94** of the vessel **88**. A distal end **110** of the conduit **106** is positioned in an inner cavity of the overflow vessel **62**. The conduit **106** is so positioned such that an amount of medium and algal culture, or another host cell type, is removed from the reaction vessel **88** at a rate substantially equal to a rate of cell growth of the algae, or another host cell type, stored in the reaction vessel **88**.

The description above applies to all of the methods and systems described herein. The following examples are for purposes of illustration only and are not intended to limit the scope of the invention as defined in the claims which are appended hereto. The references cited in this document are specifically incorporated herein by reference.

EXAMPLE 1

Inducible Plastid Expression System for a Native Gene

A vector containing the Nac2 gene under the control of the Cyc6 promoter was constructed using molecular cloning techniques known to those skilled in the art. Cloning methods are described, for example, in Sambrook et al., "Molecular Cloning: A Laboratory Manual", 3rd Edition, Cold Spring Harbor

Laboratory Press, (2001), incorporated herein by reference or in S. Surzycki, "Basic Techniques in Molecular Biology," Springer-Verlag (2000), incorporated herein by reference.

To place the Nac2 gene under the control of the Cyc6 promoter element, a chimeric DNA fragment comprising the Cyc6 promoter fused to the coding sequence of psbD was generated by overlap-extension PCR using 4 oligonucleotides specific for the Cyc6 promoter element and Nac2 genomic DNA. The resulting PCR fragment consisted of a 428 base-pair fragment of Cyc6 promoter sequence fused in frame with an 833 base-pair fragment of Nac2 genomic sequence. The PCR fragment was sub-cloned and sequenced. The PCR fragment was then cloned into the pNac2(midi) plasmid using the unique restriction sites XbaI and AatII. The pNac2(midi) plasmid contains a 5.1 kb chimeric midi-gene of Nac2 which has been previously described by Boudreau et al. (2000), incorporated herein by reference. The gene is composed of the 5' genomic sequence of Nac2 fused to the 3' cDNA sequence and results in an open reading frame (ORF) encoding the entire Nac2 protein which is tagged with a triple HA epitope at the C-terminal end of the protein. The pNac2 (midi) was digested with XbaI and AatII. The PCR fragment was then ligated directionally into the plasmid. The resulting plasmid pcy6Nac2(midi) contains a 428 basepair fragment of Cyc6 promoter sequence fused in frame with the Nac2 midi gene.

Finally, the 5.8 kb cyc6Nac2transgene was cloned into the pSL17 plasmid using the unique restriction sites in the multiple cloning site of pSL17 (i.e., EcoRI and XbaI; see FIG. **9**). pSL17 contains the aphVII cassette conferring resistance to the antibiotic paromomycin and a multiple cloning site for cloning. The resulting 10.8 kb plasmid, pcy6Nac2(paroR) (see FIG. **10**), was used to transform nac2-26 mutant cells. Sequences for Nac2 and Cyc6 used in the construction of the vector are provided in FIGS. **11** and **12**, respectively.

The pcy6Nac2(paroR) vector was introduced into a nac2 null mutant, nac2-26 by electroporation. Algal and plant transformation methods are known to those skilled in the art and are described, for example, in Sambrook et al., "Molecular Cloning: A Laboratory Manual", 3rd Edition, Cold Spring Harbor Laboratory Press, (2001), incorporated herein by reference.

To isolate a transgenic *Chlamydomonas* strain containing a Cyc6 inducible Nac2 gene, nac2-26 cells were first treated with autolysin and then transformed by electroporation using pcyc6Nac2(paroR). The resulting transformants were plated on TAP medium supplemented with the antibiotic paromomycin (20 μg/ml). Paromomycin resistant colonies were screened for the ability to grow photo-autotrophically on minimal medium supplemented with 150 μM Nickel(II), (an inducer of Cyc6 transcription) and minimal medium lacking copper (HSM-Cu^{+2}; see FIG. **13**). Photo-autotrophic strains were then tested for the ability/inability to grow on minimal medium lacking the inducer (HSM).

Using this regimen, the transgenic strain cy6Nac2.49 was isolated (see FIG. **13**). This strain was capable of growing photoautrophically on HSM-Cu^{+2} but, was unable to grow phototrophically on HSM supplemented with copper. The resulting strain, cy6Nac2.49, grows in two ways including 1.) photo-autotrophically in medium lacking copper or 2.) in anaerobically grown cultures when oxygen and copper are present in the growth medium where the cells lack PSII complexes (see FIG. **1**). Thus, in the transgenic strain, cy6Nac2.49, the production of photosynthetic oxygen is controlled through a nuclear promoter, which responds to hypoxia.

Furthermore, sealed cultures of cy6Nac2.49 grown under non-inducing conditions will quickly become anaerobic, because photosynthetic oxygen is not released but oxygen consumption by mitochondrial respiration remains constant. In sealed cultures of cy6Nac2.49, a feedback inhibition loop exists where hypoxia induces photosynthetic oxygen production, which then re-represses PSII synthesis and photosynthetic oxygen evolution.

In fact, this is exactly what was observed when sealed illuminated cultures of cy6Nac2.49 were grown in complete medium. Initially, the oxygen content of the culture was present at atmospheric levels, and then it was quickly consumed, resulting in anaerobis and induction of hydrogen evolution. After a period of anaerobic growth, oxygen returned to the culture at 2 times the atmospheric levels inside the vessel, inhibiting hydrogen evolution (data not shown). In a conventional sealed vessel, only one cycle of oxygen consumption and hydrogen production was observed, possibly because the consumption of oxygen ceased when the reduced carbon was consumed by mitochondrial respiration while photosynthetic oxygen production remained constant.

Wild-type, nac2-26 (the parental strain of cy6Nac2.49) and, cy6Nac2.49 cells were tested for their ability to grow on complete (TAP) medium, complete medium supplemented with the antibiotic paromomycin (TAP+Paro), minimal medium (HSM), and minimal medium lacking copper (HSM-Cu^{+2}) (FIG. **10**). Western blot analysis performed on whole cell extracts of wild-type and cy6Nac2.49 cells grown in non-inducing (TAP) and inducing conditions (TAP-Cu^{+2} and TAP-O_2) is shown in FIG. **14**. Western blots were probed with antibody recognizing α-HydA as a control for anaerobiosis, α-D2 and α-RbcL.

FIG. **15** shows the result of an experiment to measure hydrogen evolution. Cy6Nac2.49 cells were sealed in an illu-

minated vessel where dissolved gas in the culture medium could be measured using a mass spectrometer (oxygen-dashed line, hydrogen-solid line), then shifted from inducing to non-inducing conditions through the addition of copper to the growth medium (TAP-Cu^{+2} to TAP). The concentration of H_2 was measured in the liquid phase and is indicated in mBar.

Conventional sealed vessels are not adequate to sustain hydrogen production using the transgenic strain cy6Nac2.49, so a sealed, anoxic system was designed to provide fresh, oxygen-depleted medium at a constant rate to a growing cy6Nac2.49 culture, to maintain it in exponential growth phase (see FIGS. **7** and **8**). It was hypothesized that maintaining a culture in an exponential growth phase would result in the establishment of a cycle of oxygen consumption/hydrogen production/oxygen production. When cells of cy6Nac2.49 were grown in this system, we observed an induction of hydrogen evolution shortly after the onset of hypoxia to the culture. Synthesis of PSII complexes was then induced along with a slight rise in oxygen in the vessel. Unlike the conventional sealed vessels, the photosynthetic oxygen released does not inhibit hydrogen production, presumably because photosynthetic oxygen production never exceeds the consumption of oxygen by mitochondrial respiration. In this system, the production of hydrogen is directly linked to light energy and therefore represents a direct biophotolysis method for the production of hydrogen. Using the anoxic system, we achieved a constant rate of hydrogen evolution reaching approximately 0.5% of the gas phase.

Oxygen is evolved as a byproduct of photosynthesis. As a result, a central challenge to sustaining algal hydrogen evolution using light energy (a process sometimes known as biophotolysis) has been to overcome oxygen sensitivity of the hydrogenase enzyme. In contrast to the direct biophotolysis method developed by these inventors, indirect biophotolysis methods (or two-stage photosynthesis and hydrogen production) apply spatial or/and temporal separation of the photosynthetic oxygen and hydrogen production to overcome the oxygen-sensitivity of the hydrogenase enzyme (Benemann 1996; Melis 2000). The first stage involves normal oxygenic photosynthesis: the release of oxygen, fixation of CO_2, and accumulation of biomass. In the second phase, oxygenic photosynthesis is inhibited physiologically through the depletion of an important nutrient such as sulfur. Because rates of oxygenic photosynthesis decline drastically after about 22 hours of sulfur starvation, sealed cultures become anaerobic owing to the net consumption of oxygen caused by mitochondrial respiration (Melis 2000). Once anaerobia is established, the hydrogenase pathway is induced and hydrogen is evolved using electrons derived primarily from the remaining photooxidation of water, but also from the catabolism of endogenous substrates such as protein and starch (Ghirardi 2000).

The differences between the anoxic system, a direct biophotolysis method, and indirect biophotolysis methods reveals several important advantages for the use of the anoxic system. First, inherent to the anoxic system, there is a 50% gain in capacity for hydrogen production simply because oxygen and hydrogen production occur in a single illuminated sealed vessel. The two-stage photosynthesis and hydrogen production method or indirect biophotolysis implies the temporal and/or spatial separation of the oxygen and hydrogen production phases and as a result, requires two vessels with one of the vessels not being used during hydrogen production with a loss of 50% of capacity for production. Secondly, two-stage photosynthesis and hydrogen production methods rely on physiological depletion of sulfur to inhibit oxygenic photosynthesis. Severe sulfur starvation has a wide range of effects on a variety of cellular processes. The hydrogenase enzyme that catalyzes the release of hydrogen contains Fe—S clusters, assembly of which is required for its function (Posewitz, et al. (2004)). In addition, sulfur depletion negatively affects other important chloroplast complexes important to hydrogen evolution like PSI. Clearly, physiological depletion of sulfur severely affects important parts of the hydrogen evolution machinery. In contrast, the anoxic system does not rely on depletion of an important micronutrient to induce hydrogen production. In fact, hydrogen production occurs under optimum physiological conditions (in an exponentially growing culture in complete medium).

Finally, a major hurdle in the large-scale production of hydrogen using, for example, algae is providing light-energy to large dense cultures. In large dense cultures of algae, large quantities of chlorophyll in the light-harvesting complexes can prevent light energy from reaching cells at the center of the vessel. In our direct biophotolysis method, hydrogen production occurs in a system, where an optimum cell density can be established for maximum light absorption and hydrogen production.

EXAMPLE 2

Inducible Plastid Expression System for a Foreign Gene

Chloroplast transformation vector. The plasmid pSK108 contains a 3 kb fragment of chloroplast DNA which includes the psbD gene and 5' flanking sequences to direct it to the region surrounding the psbD gene (FIG. **16**). The construct also contains the aadA cassette inserted upstream of the psbD gene. A transgene can be inserted in frame using the NcoI and SphI sites of pSK108. Once ligated, the new vector would contain the transgene (plus 3 bps) with the 5' end of atpA driving its expression and the 3' sequence of rbcL acting as a terminator. The atpA promoter drives the expression of a gene encoding the ATP-generating proton pump of the chloroplast, and, thus, is not subject to the D1 repair mechanism.

Three FLAG-tagged foreign genes wVP28, DILP, and IBVD were individually subcloned into the chloroplast transformation and expression vector pCG12 (FIG. **17**). The vector integrates downstream of the atpB chloroplast locus. The transgenes are driven by the atpA promoter and carry the stability factor association site of the 5' UTR of psbD (FIGS. **18** and **19**).

These vectors were co-transformed with the p228 vector, which carries the 16S rRNA gene of *Chlamydomonas* and confers resistance to spectinomycin, into the cyc6/Nac2 inducible strain (Cyc6 promoter as the control promoter and Nac2 as the stability factor).

EXAMPLE 3

Overexpression of the HydA1, HydEF, and HydG Genes in *C. reinhardtii* Chloroplasts

Whether hydrogenases can be overexpressed in *C. reinhardtii* and whether this leads to enhanced hydrogen production will be tested. Recent experiments have shown that coexpression of HydEF, HydG and HydA1 in *E. coli* is sufficient for producing active Fe hydrogenase (Posewitz, et al, (2004)). Overexpression of proteins in the nuclear compartment of *C. reinhardtii* has met with little success mostly because transgenes are often silenced. Therefore, these genes will be expressed in the chloroplast compartment using biolistic transformation. There are several advantages for using this strategy. First, gene silencing does not occur in the chloro-

plast. Second, each plastid gene is present in 80 copies in *C. reinhardtii*. Third, recent experiments have shown that it is indeed possible to achieve high expression levels of foreign proteins in the chloroplast if these genes are driven by chloroplast promoters, 5' and 3' UTRs, and if the coding sequence is resynthesized using biased chloroplast codon usage (Mayfield, et al. (2001)). There is a strong AT bias for chloroplast genes.

Each of the coding sequences of HydA1, HydEF and HydG will first be reconstructed taking into account the chloroplast codon usage of *C. reinhardtii*. This will be achieved by using published methods which have been used successfully for synthesizing foreign genes for expression in the chloroplast. As an example, we have recently successfully overexpressed two viral proteins in the chloroplast of *C. reinhardtii* (see below). The transgene will be inserted in the chloroplast inverted repeat so as to increase its copy number twofold. As a host strain, the cyc6Nac2 strain will be used. This strain contains the nuclear nac2 mutation and the Nac2 gene driven by the Cyc6 promoter which is induced by copper depletion or anaerobic conditions.

At this time it is not known which protein among the HydA1p, HydEFp and HydGp proteins is limiting for hydrogen production. To test this, each of the three genes fused to the psbD promoter and 5' UTR will first be inserted individually within the chloroplast inverted repeat using biolistic transformation for overexpression. One possibility is to insert the gene in the ribosomal operon within the spacer between the 16S and 23S rRNA genes, a strategy which has been used successfully for high expression in the chloroplasts of higher plants. Because the transgene is under control of the psbD 5' UTR which is driven via Nac2 by the Cyc6 promoter, the transgene will only be expressed under anaerobic conditions. Expression will be monitored by RNA blot hybridizations or real time RT-PCR. For each of the three genes tested, hydrogen production will be assayed by growing the transformed cells in a closed bottle connected with a tubing to an upside-down burette filled with water and by measuring the volume of the gas directly from the volume of water that is displaced as described by Zhang and Melis (2002). If hydrogen production is increased relative to the cyc6Nac2 control, this will indicate that the overexpressed protein is limiting. If these experiments reveal that more than one of these proteins is limiting for hydrogen production, both proteins will be expressed using the same strategy. It is possible that expression of these three proteins is adjusted in wild-type cells so that each protein needs to be overexpressed to increase the yield of hydrogen production. In this case overexpresson of the three proteins will be required. Expression of multiple transgenes has been achieved in higher plant chloroplasts (Quesada-Vargas, et al. (2005)). If expression with the psbD 5'UTR is not sufficient, other strong chloroplast promoters-5'UTRs such as psbA, atpA and the ribosomal promoter will be tested.

EXAMPLE 4

Expression of Genes that Affect Hydrogen Production

In addition to the above, several other genes will be tested for increased hydrogen production using the inducible and/or repressible system for the expression of proteins in plastids. Two examples are oxygen-insensitive hydrogenases or reducing antenna size (Melias, et al. (2004); Ghirardi, et al. (2005)). Briefly, the former involves cloning and characterizing native and mutagenized hydrogenase genes from *Chlamydomonas* and other organisms for reduced sensitivity to oxygen. The objective of the latter, antenna reduction, is to reduce the amount of photons captured by algal cells, so that light may penetrate deeper into photoreactors, thus becoming available for use by normally shaded cells. Algal cells are very effective at capturing but not utilizing light, wasting as much as 80% of absorbed photons. Genes that regulate antenna size have been identified through DNA insertional mutagenesis. The PSII and PSI antenna size of mutant, tla1, was 50% and 65% of the wild type strain. Finally, mutants strains have been identified with presumably increased rates of respiration that consequently lower the levels of oxygen available for inhibiting hydrogenases (Krause, et al. (2005)). The genes will be inserted into the cy6Nac2.49 transgenic strain and evaluated for their contribution to increasing hydrogen production of the strain.

One important limiting factor for hydrogen production is the competition with the Calvin-Benson cycle. One possibility to enhance electron flow to the hydrogenase is to decrease the activity of the Calvin-Benson cycle by decreasing the amount of an enzyme which participates in this cycle. For example, phosphoribulose kinase (PRK) could be utilized because two mutants of *Chlamydomonas*, F60 and ac214, deficient in PRK activity are available. The PRK gene will be fused to the inducible Cyc6 promoter and this construct will be inserted into F60. The advantage of this strategy is that PSII and the Calvin-Benson cycle will be operational together in the absence of copper (or oxygen) thus allowing for storage of reducing power. Both genes will be shut off in the presence of copper (or oxygen). Thus, under conditions in which hydrogenase is induced, electron flow will be diverted from the Calvin-Benson cycle to the hydrogenase and hydrogen production will be enhanced.

In another illustrative embodiment, PRK temperature-sensitive mutants will be screened for. In this way the Calvin-Benson cycle can be shut off at the restrictive temperature and electrons diverted to the hydrogenase. The PRK mutants will be transformed with a library of mutagenized PRK genes generated by PCR. Transformants will be selected on minimal medium at the permissive temperature (24° C.). Colonies will be replica-plated to minimal medium and will be grown at the restrictive temperature (32° C.) and mutants unable to grow will be identified. To verify that the PRK gene carries a mutation, the gene will be amplified by PCR and sequenced. It will also be possible to screen the mutants by fluorescence since a block in the Calvin-cycle is likely to increase the fluorescence yield of these mutants.

In another embodiment, cyclic electron flow represents another route of diversion of electrons from the hydrogenase, and we will use mutants deficient in state transitions that are blocked in state 1. State transitions involve a rebalancing of the light excitation energy between the antenna of PSII (LHCII) and PSI through a reversible displacement of the mobile part of LHCII from PSII to PSI under changing light conditions which allows for an optimal photosynthetic yield. In state 1, the mobile part of LHCII is associated with PSII, whereas in state 2 it is associated with PSI. Moreover, in *Chlamydomonas*, state 1 favors mostly linear electron flow while state 2 leads to cyclic electron flow (Finazzi et al., 2002). Thus, there is no cyclic electron flow in mutants blocked in state 1. The stt7 mutant which is known to be blocked in state 1 (Depège et al., 2003) will be crossed with nac2-26 containing the Cyc6-Nac2 and Cyc6-PRK construct and it will be determined whether this leads to improved hydrogen production when PSII activity is repressed. Alternatively, once the temperature-sensitive PRK mutants are available, the mutations will be crossed to the nac2-26 stt7

Cyc6-Nac2 strain. These strains will be tested for hydrogen production under the conditions described above.

Other mutants besides stt7 deficient in state transitions will be analyzed. The analysis of these mutants is of particular interest because alterations in state transitions can be caused indirectly by alterations in the regulation of photosynthetic and mitochondrial electron flow which may change the ratio of mitochondrial respiration relative to photosynthetic oxygen evolution. Mutants of this sort have already been reported to have increased hydrogen production in comparison to WT cells (Kruse et al., 2005).

EXAMPLE 5

Inducible Plastid Expression System for Foreign Genes

The selectable marker gene, aadA, was used for inducible chloroplast gene expression. It can be expressed in a Nac2-dependent fashion, can be easily screened using the antibiotic spectinomycin, and it is known to have a high specific activity. Even low aadA expression results in some antibiotic resistance, and therefore provides a measure for the "tightness" of cy6Nac2 regulation in Ind41__18 (described below). A chloroplast integration vector had been constructed that carries the promoter and 5'UTR of psbD driving the expression of the aadA gene.

The nac2-26 mutant strain was previously described (Kuchka, et al. EMBO J. 7, 319-324; Nickelsen, et al. EMBO J. 13, 3182-3191). The cyc6Nac2.49 strain contains a transgene consisting of the Cyc6 promoter fused to the Nac2 midi-gene inserted into the nuclear genome of the nac2-26 mutant (Δnac2::cy6proNac2). Ind41 was derived from cy6Nac2.49 by replacing the psbD promoter and 5'UTR with a 675 fragment containing the petA promoter and 5'UTR (Δnac2::cy6pro Nac2::5'petA-psbD). Ind41-18 is related to the Ind41 strain, except that the aadA cassette in the Ind41-18 strain has been completely excised from the chloroplast DNA and the strain is therefore sensitive to spectinomycin (Δnac2::cy6proNac2::5'petA-psbD[Spc^S]). Ind_aadA__117 was derived from Ind41-18 and contains the aadA cassette driven by the psbD promoter and 5'UTR inserted downstream of the atpB gene (Δnac2::cy6proNac2::5'petA-psbD::5'psbD-aadA).

Screening for Ind_aadA Transgenic Strains

Integration of the 5' psbD-aadA cassette into the chloroplast genome of Ind41__18 cells was accomplished through biolistic transformation of copper-starved Ind41__18 cells with the pcg12 chloroplast integration vector (FIG. **21**). Selection of the transformants was on copper-depleted TAP medium containing 100 μg/ml spectinomycin. Colonies resulting from the transformation were picked and re-plated three times on TAP-Cu^{+2} medium supplemented with 100 μg/ml spectinomycin to ensure complete segregation of the 5'psbD-aadA cassette.

A schematic diagram of the pcg12 vector used in the experiment is shown in FIG. **21**. The aadA cassette in the vector is expressed using the psbD 5' UTR. Growth of wild type, Ind41__18, and Ind_aadA_X transgenic strains is also shown. The strains were serially diluted, then spotted on solid TAP, copper-depleted TAP media (TAP-Cu^{+2}), TAP supplemented with 100-1000 μg/ml spectinomycin (TAP-Cu^{+2}+Spc), and TAP-Cu^{+2} supplemented with 100-1000 μg/ml spectinomycin (TAP-Cu^{+2}+Spc) and cultured for 7-10 days at a light intensity of 100 μE $m^{-2}s^{-1}$.

Screening of transformants was accomplished by replica plating the putative inducible aadA transgenic lines on TAP+Spc100 and TAP-Cu^{+2}+Spc100 solid agar medium. All of the colonies tested grew equally well on both culture media, indicating a loss of promoter control of the Cyc6Nac2 transgene in these strains (FIG. **21**A). However, when higher concentrations of spectinomycin were tested, 85% of the strains tested were sensitive to spectinomycin when copper was supplemented in the growth medium, but not when copper was omitted from the medium (FIG. **21**B). Several of these strains, named Ind_aadA, were chosen for further characterization. The genotype of these strains is nac2::$cy6_{Pro}$Nac2::5'petA-psbD::5'psbD-aadA.

Growth Analysis of Ind_aadA Transgenic Strains

Growth analysis of several inducible strains retained from the initial screening process was accomplished by serial diluting, then spotting wild-type, Ind41__18 and Ind aadA cells on either TAP, TAP-Cu^{+2}, HSM, HSM-Cu^{+2} and solid agar medium supplemented with a range of spectinomycin concentrations (0, 100, 250, 500, 750, 1000, 2000 μg/ml) (FIG. **21**B). Of the 11 strains tested in this fashion one, Ind_aadA__36 was capable of growing on TAP medium supplemented with >500 μg/ml spectinomycin. On the other hand, 6 of 11 strains tested were able to grow on copper-depleted TAP medium at low spectinomycin concentrations but could not grow with spectinomycin concentrations above 250 μg/ml (FIG. **21**B). One strain named Ind_aadA__117 was sensitive to spectinomycin when cultured on TAP +250 μg/ml spectinomycin, but was capable of growing on copper-depleted TAP medium at all concentrations of spectinomycin tested.

Northern Analysis of Total RNA Extracted from Ind_aadA__117

To gain insight into the induction of aadA expression in Ind_aadA__117, total RNA was isolated from Ind_aadA__117 cells cultured in either non-inducing (TAP) or inducing conditions (TAP-Cu^{+2}, TAP-Cu^{+2} +Spc, TAP-O_2, HSM-Cu^{+2}). Northern analysis of these samples revealed that psbD transcripts accumulated to the same levels as was described for the parental strain, Ind41__18 or approx. 25% of the wild-type psbD RNA. As expected, the psbD RNA of Ind_aadA__117 was larger than wild-type psbD transcripts, indicating that the authentic psbD gene is no longer present in this strain, a characteristic inherited from the parental strain, Ind41__18 (FIG. **22**). Importantly, accumulation of aadA transcripts was observed in all cultures in which Cyc6 transcription was induced (FIG. **22**). The "leaky" phenotype of the Ind_aadA strains was confirmed by the presence of a small amount of aadA RNA in TAP grown Ind_aadA__117 cultures (FIG. **22**). Surprisingly, aadA transcripts were more abundant in anaerobically grown cultures than copper-starved cultures of Ind_aadA__117, a characteristic not shared by the "grand-parental" strain cy6Nac2.49 with respect to psbD/D2 expression (FIG. **22**). In the assay shown in FIG. **22**, total RNA was extracted from wild-type, and Ind_aadA__117 cells cultured in TAP, TAP-Cu^{+2}, TAP-Cu+2+Spc TAP-O_2 +Spc, HSM-Cu^{+2} and HSM-Cu^{+2}+Spc liquid media. Probes are indicated on the left in FIG. **22**.

Analysis of Ind_aadA__117 Protein Extracts

Total proteins extracted from Ind_aadA__117 cells were analyzed by immunoblots using antisera specific for several important chloroplast-encoded proteins. D2 protein levels were found at approximately 25% of wild-type levels in all cultures of Ind_aadA__117 examined, less than in the parental strain Ind41__18 which accumulated 50% of D2 protein (FIG. **23**). On the other hand, full restoration of D1 and CP47 protein accumulation was observed in most of the Ind_aadA__117 protein extracts tested and, in this case, D2 protein accumulation was estimated to be reduced by 75%. Therefore, surprisingly, D1 and CP47 accumulation was not

adjusted to D2 protein levels. Nac2 protein accumulation in Ind_aadA_117 was determined using soluble extracts prepared from Ind_aadA_117 cultured under a variety of conditions. Induction of Cyc6Nac2 was observed in soluble extracts from anaerobic and copper-starved Ind_aadA_117, although, a small amount of Nac2 was detected in TAP grown Ind_aadA_117 cultures (FIG. **23**). The presence of trace amounts of Nac2 in TAP cultures, a condition where the transcription of Cyc6 is repressed, demonstrated that the system was slightly "leaky" in the Ind aadA strains (FIG. **23**). Therefore, the initial observation that Ind_aadA transgenic strains cultured on TAP medium were able to grow in the presence of low concentrations of spectinomycin is due to some leakiness of Cyc6Nac2. In FIG. **23**, total proteins were extracted from wild-type, and Ind_aadA_117 cells cultured in TAP, TAP-Cu^{+2}, TAP-Cu+2+Spc TAP-O_{2+}Spc, HSM-Cu^{+2} and HSM-Cu^{+2}+Spc liquid media and were size fractionated and immobilized on PVDF membranes. Probes are indicated on the left of FIG. **23**.

aadA Assay of Ind_aadA_17

The amount of aadA was estimated indirectly by measuring its activity (Table 1). The assay is based on the ability of the aadA enzyme to transfer the adenyl moiety of an ATP molecule to spectinomycin, thereby adding a positive charge to the spectinomycin molecule. Positively charged spectinomycin molecules can bind a phospho-cellulose membrane that carries a negative charge. Thus, if crude extracts of *Chlamydomonas* expressing aadA are incubated in the presence of spectinomycin and α^{32}P-labeled dATP, the amount of radioactivity present on the phospho-cellulose membranes after spotting the reactions and washing off the non-specific products provides a relative measure of the activity of the aadA enzyme. Crude extracts of Ind_aadA_117 cells cultured in either TAP, TAP-Cu^{+2}, TAP-Cu^{+2} +Spc liquid media were used to measure the aadA activity in this transgenic strain compared to the parental strain and another strain expressing the traditional aadA cassette in the wild-type background. The combined results of several independent assays are presented in Table 1. No aadA activity was detected in Ind41_18 thus demonstrating that the removal of the cassette in this strain is complete. On the other hand, aadA activity in both the wild-type strain expressing the traditional aadA cassette, and in Ind_aadA_117 grown in TAP-Cu^{+2} and TAP-O_2 was similar to that previously reported for aadA-expressing transgenic strains (Table 1). A small but significant aadA activity was also present in crude extracts of Ind_aadA_117 cultured in TAP medium. This result confirmed, that a small amount of aadA activity was present in uninduced Ind_aadA_117, although the activity was only a fraction of the activity of induced cultures of Ind_aadA_117.

Screening for strains capable of inducing the expression of the aadA gene using the cy6Nac2 chloroplast inducible gene expression system consistently resulted in the recovery of strains that had a "leaky" phenotype. Because the Cyc6Nac2 transgene was demonstrated to be tightly regulated in the parental strain, de-repression of Cyc6Nac2 transgene in Ind_aadA was, by inference, considered a prerequisite for surviving the screening process. One possible explanation for this observation is that there was initially a very small amount of copper contamination in the copper-depleted medium used at each step in the screening process, including in the media used for the biolistic transformation of Ind41_18 with pcg12. If this was indeed the case, a transient repression of Cyc6 transcription would be predicted to occur until copper contamination dropped below 2×10^6 ions/cell, and as a result, only those transformants that de-repressed Cyc6Nac2 would survive the original transformation with the pcg12 plasmid. In other words, given that repression of Cyc6Nac2 transcription was long enough to negatively effect cell survival in spectinomycin-supplemented media; all colonies that survived the original transformation were generated from a single cell that would not have divided unless Cyc6Nac2 had been de-repressed. Contamination of the culture medium was considered to be unavoidable as it has been well documented that even minute concentrations of copper repress the transcription of Cyc6. Indeed experiments designed to study the kinetics of copper-mediated repression in cy6Nac2.49 revealed that re-suspension of copper-starved cy6Nac2.49 cells in copper-depleted media, transiently repressed Cyc6Nac2 transcription, as well as transcription of the authentic Cyc6 locus for 1-2 cycles of division.

Nevertheless, the Ind_aad_117 transgenic strain shows that the design for an inducible chloroplast gene expression system based on the nucleus-encoded Nac2 protein is possible for *Chlamydomonas*. The transgenic Ind41_18 strain can be used to induce the expression of any chimeric gene driven by the psbD 5'UTR integrated in the chloroplast genome, provided that the psbD 5**402** UTR is capable of driving the expression of the gene of interest.

For the assays in Table 1, aminoglycoside adenyl transferase activity in Ind41_aadA-117 was determined under inducing and repressing conditions. Extracts from WT-aadA, Ind41_18 and Ind41_aadA-117strains were assayed for aadA activity and for total protein content. The activity is indicated as cpm incorporated per μg of protein. Numbers of independent measurements are indicated in parenthesis.

TABLE 1

Strain	+Cu	−Cu
WT	1.4 +/− 2.0	nd
WT-aadA	207.0 +/− 49.5 (3)	192.6 +/− 51.4 (4)
Ind41_18	9.2 +/− 4.1 (4)	12.2 +/− 7.9 (4)
Ind41_117	24.2 +/− 12.5 (4)	274.3 +/− 90.6 (7)

Inducible Expression of the VP28_FLAG, IBVD_FLAG, and DILP_FLAG

To test if the inducible chloroplast gene expression system described here could be applied to the production of foreign proteins, three different foreign proteins were selected for heterologous expression using the 5' psbD driven transgenes, VP28, DILP, and IBVD. IBVD (or VP2) is used to generate a vaccine for the control of Infectious Bursal Disease Virus (IBDV) in poultry (Mundt 1995). VP28, the 23 kD fragment of a major structural envelope proteins of White spot syndrome virus of Penaeus monodon, was demonstrated to protect shrimp from infection when fed as a subunit vaccine (Witteveldt 2004).

Three FLAG-tagged foreign genes VP28, DILP, and IBVD with a codon bias that was optimized for the *C. reinhardtii* chloroplast genetic machinery were provided as a gift from Dr. Stefan Surzycki (Indiana University, Bloomington) and were individually subcloned in the chloroplast transformation and expression vector pcg12 so that the 5'UTR of psbD was driving the expression of the foreign protein with the rbcL 3'UTR as a terminator (FIG. **24**A). Because these new constructs lacked a selectable marker, the generation of transformants required co-transformation with another chloroplast integration vector carrying a selectable marker (FIG. **24**A).

A schematic diagram of the pcg12 _DILP vector used in experiments to induce the expression of Drosophila insulin-like peptide is shown in FIG. **24**A. The black box represents DILP coding sequence. The arrow represents the 5' leader of

the psbD gene. The asterisks indicate insertion of the 3HA-11 epitope. In FIG. **24**B, immunoblots using total proteins extracted from Ind_VP28 (upper left), Ind_IBVD (upper right) or Ind_DILP (lower panel) are shown, probed with antibody that recognized the FLAG epitope. The predicted molecular weight of the proteins are VP28-23 kD, IBVD-49 kD, and DILP-12 kD and are indicated with an asterisk.

These vectors were co-transformed with the pY1_INT vector, which carries the ycf1 gene and flanking chloroplast sequence and the aadA cassette which confers resistance to spectinomycin into the Ind41_18 strain. Following selection on TAP plates amended with spectinomycin, putative transformants were tested for the presence of the gene using PCR with oligonucleotides that amplified the foreign gene. Of the 10 colonies tested by PCR for co-insertion of the transgene, seven were positive for VP28 and DILP and five were positive for the presence of the IBVD gene. These lines were named IndVP28_x, IndDILP_x and IndIBV_x and had the genotype of nac2.Cyc6$_{pro}$Nac2::5'petA-psbD::5'psbD-VP28/DILP/IBVD.

Colonies that tested positive for the insertion of the foreign gene in the chloroplast genome were tested for protein production using immunoblot analysis with the Flag® antibody following induction of the gene by copper starvation. Of the 22 transgenic lines tested in this way, 8 of 8 appeared to accumulate the VP28 protein, as a 23 kD protein was induced in extracts from IndVP28 that was not present in either the wild-type or the non-induced controls (FIG. **24**B—Upper left). Total protein extracted from 4 of 6 IndDILP strains analyzed also accumulated a >25 kD protein when these cells were induced (FIG. **24**B—Lower panel). A 50 kD protein accumulated in 3 of 7 IndIBVD strains assayed in this way (FIG. **24**B—Upper right).

FIG. **25** shows based on immunoblots with total proteins extracted from IndDILP strains that the expression of DILP_FLAG was induced in initial screening experiments. IndDILP strains were cultured in either TAP (ui) or TAP-Cu^{+2} (i) liquid media supplemented with 50 μg/ml spectinomycin and size fractionated on 15% SDS-PAGE gels. The resulting immunoblots were then incubated with antibodies that recognized the FLAG epitope. Total proteins extracted from a transgenic strain that expressed a FLAG-tagged Alb3.1 protein were used as a positive control for the detection of the FLAG epitope in the experiment. The asterisk indicates predicted size of the DILP_FLAG peptide inserted in the pcg12_DILP vector.

Proteins extracted from three IndDILP lines shown to express the DILP protein were characterized further by comparing the proteins extracted from these strains when Cyc6 transcription was repressed versus induced. Of the four IndDILP strains tested, two seemed to correctly induce the production of DILP_FLAG, as a 12 kD protein. Therefore, the chloroplast inducible expression system developed here induced the expression of DILP. This finding was particularly interesting as previous attempts to expression the DILP protein in a constitutive fashion in the chloroplasts of *Chlamydomonas reinhardtii* never resulted in a high level of expression (Stefan Surzycki, personal communication). Thus, the inducible chloroplast gene expression system may provide a commercially relevant tool for the inducible expression of foreign proteins, especially those proteins resistant to constitutive expression.

EXAMPLE 6

Expression of Genes that Affect Hydrogen Production

Strains and Media

The nac2-26 mutant strain was previously described (Kuchka, et al. EMBO J. 7, 319-324; Nickelsen, et al. EMBO J. 13, 3182-3191). The cyc6Nac2.49 strain contains a transgene consisting of the Cyc6 promoter fused to the Nac2 midi-gene inserted into the nuclear genome of the nac2-26 mutant (Δnac2::cy6proNac2). Ind41 was derived from cy6Nac2.49 by replacing the psbD promoter and 5'UTR with a 675 fragment containing the petA promoter and 5'UTR (Δnac2::cy6pro Nac2::5'petA-psbD). Ind41-18 is related to the Ind41 strain, except that the aadA cassette in the Ind41-18 strain has been completely excised from the chloroplast DNA and the strain is therefore sensitive to spectinomycin (Δnac2::cy6proNac2::5'petA-psbD[Spc^S]). Ind_aadA_117 was derived from Ind41-18 and contains the aadA cassette driven by the psbD promoter and 5'UTR inserted downstream of the atpB gene (Δnac2::cy6proNac2::5'petA-psbD::5'psbD-aadA). All strains were maintained on TAP (Tris-acetate-phosphate) medium supplemented with 1.5% Bacto-agar at 25° C. under dim light. In experiments where copper-supplemented or copper-deficient (—Cu^{+2}) solid agar and liquid TAP and HSM medium was employed, medium was prepared according to Quinn and Merchant (Methods in Enzymol. 297, 263-279). TAP and TAP-Cu^{+2} media was supplemented with 100 μg/ml spectinomycin (Sigma-Aldrich) or 20 μg/ml paromomycin (Sigma-Aldrich) where necessary. In experiments where cells were deprived of oxygen, liquid cultures where bubbled with N_2 gas with 150 rpm/min agitation and constant light illumination (20 $\mu E/m^{-1}s^{-2}$). Cell density was determined using a hemacytometer.

Plasmid Construction

Standard techniques were used to manipulate and analyze all plasmid constructs. Sequencing of constructs was carried out using BigDye terminator sequencing kit (Applied Biosystems, La Jolla, Calif.) and an ABI Prism 377 automated sequencing machine (ABI). The bacterial host used for cloning in *E. coli* was DH10B (Amersham Biosciences). All oligonucleotides were ordered from Microsynth GmbH (Balgach, CH).

Transformation of *Chlamydomonas* Cells

Nuclear transformation of *Chlamydomonas reinhardtii* strains nac2-26 was performed by electroporation essentially as described in Shimogawara, et al. Genetics 148, 1821-8.

Chlorophyll and Oxygen Evolution Rate Measurements

Oxygen evolution and respiration rates were determined using a Clark type oxygen electrode attached to a X type light source at 25° C. (Hansatech Instruments Ltd., Norfolk, UK).

Hydrogen Measurements and Calculations

All liquid phase and gas phase measurements of N_2, O_2, H_2 and CO_2 were performed as follows. Continuous monitoring of dissolved gases were made using a sealable, thermo-stated Clarks type vessel where gases were fed into the ion source of a mass spectrophotometer (model MM 880; VG Instruments, Cheshire, United Kingdom) through a polypropylene membrane under continuous agitation and constant illumination using a fiber-optic illuminator (model KL 1500, Schott, Mainz, Germany). In experiments where transcription of the Cy6Nac2 transgene was repressed, 12 μM copper was added to the growth medium within the vessel (TAP-Cu^{+2} to TAP). Calibration of the mass spectrometer before all gas phase time-points was achieved through injection of air and pure hydrogen gas samples directly into the ion source.

Assay for aadA Activity

Assays for aadA activity were carried out on wt, Ind41-18, and Ind_aadA_117 strains essentially as described in Goldschmidt-Clermont, Nucleic Acids Res 19, 4083-9. except that ^{32}P-labeled dATP was used in place of the radiolabeled rATP used in the original experiments.

Plasmid Constructions—pRS1_rcy_aadA Construction

The plasmid pKS-108#14 was used for the construction of plasmid pRS1_rcy_aadA. The pKS-108#14 plasmid contains the chloroplast DNA EcoRI R3 fragment of *C. reinhardtii*, with the aadA cassette inserted at position −263 bps relative to the psbD ATG initiation codon. In order to replace the psbD 5'UTR with the petA 5'UTR, a 943 by chimeric DNA fragment comprised of the petA 5'UTR fused to the coding sequence of psbD was generated using overlap-extension PCR with oligonucleotides RS1, RS2, RS3 and RS4 (Table2). The resulting PCR product was digested with PvuII/ClaI restriction endonucleases and ligated into plasmid pKS-108#14 digested with the same enzymes to generate the pRS1 plasmid. The design of the chloroplast inducible expression system involves two successive chloroplast transformations which would require two different selectable markers. Alternatively, recycling of aadA is possible using a modified aad4 cassette that is flanked by 483 by direct repeats, allowing for the efficient removal of the cassette by homologous recombination after the selective pressure has been removed. Cloning of the recyclable aadA cassette into pRS1 was achieved by first cutting pRS 1 with ClaI and SphI, and then filling the 5' and 3' ends of the resulting 6.3 kb plasmid with T4 DNA polymerase. This resulted in the excision of the atpA promoter and 5'UTR fused to the aadA coding sequence from the pRS1 plasmid, but did not remove the 3' sequence of rbcL. Insertion of the recyclable cassette into pRS1ΔaadA was achieved by first excising the recyclable aadA cassette from pKS-483-aadA-483 plasmid using SacI and KpnI restriction endonucleases and blunting both ends with T4 DNA polymerase and PNK kinase. Blunt end ligation of the 2.8 kb recyclable aadA cassette into pRS1ΔaadA was accomplished to generate pRS1_rcy_aadA (FIG. **27**).

Construction of pcy6Nac2(paroR)

To construct a plasmid with the 428 by Cyc6 promoter sequence fused in frame with the Nac2 coding sequence, a 5.1 kb chimeric midi-gene of Nac2 was employed. In brief, the plasmid pKS(−)nac2(midi) contains 3.0 kb of 5' Nac2 genomic sequence ending at the SfrI restriction site within the Nac2 coding sequence fused to a 1.96 kb Sfr1/XhoI fragment containing the 3' cDNA sequence tagged with 3 HA, 6 His (SEQ ID NO: 1) and 9 Myc epitopes, introduced in frame with the Nac2 coding sequence just upstream of the stop codon. In order to place the Nac2 gene under the control of the Cyc6 promoter, a chimeric DNA fragment comprising the Cyc6 promoter fused to the coding sequence of Nac2 was generated by overlap-extension PCR using 4 oligonucleotides specific for the Cyc6 promoter element and Nac2 genomic DNA. The resulting PCR fragment consisted of the 428 by Cyc6 promoter fragment fused in frame with an 833 by genomic Nac2 fragment. The PCR fragment was then cloned into the pNac2 (midi) plasmid using the unique restriction sites XbaI and AatII of pKS(−)Nac2(midi). Finally, the 5.8 kB Cyc6Nac2 trans-gene was cloned into the pSL17 plasmid using the unique sites EcoRI and XbaI of pSL17. This plasmid contains the aphVII cassette conferring resistance to paromomycin. The resulting 10.8 kb plasmid, pcy6Nac2(paroR), was used to transform nac2-26 mutant cells.

Transformation of *Chlamydomonas* Cells

Cells of nac2-26 cells were grown in TAP medium, harvested in mid-log phase (2-4×10^6 cells/ml), and treated with gamete autolysin, then re-suspended in TAP+40 mM sucrose medium. For each electroporation, 10^8 treated cells were incubated with 2.5 linearized pcyc6Nac2(paroR) or pSL17 plasmid DNA (to determine electroporation efficiency), plus 50 μg salmon sperm DNA, then transformed by electroporation in a 0.2 ml electroporation cuvette (Biorad, USA) using the Biorad (SIC) set to 0.75 kV, 25 μF and no resistance (Biorad, USA). The treated cells were recovered in 1 ml fresh TAP, 40 mM sucrose, 0.4% PEG-8000, 20% starch medium for 10 minutes, and plated on TAP medium supplemented with the antibiotic paromomycin (20 μg/ml). Paromomycin resistant colonies were screened for the ability to grow photoautotrophically on minimal medium lacking copper (HSM-Cu^{+2}) at 25° C. in high light (45 $\mu Em^{-2}s^{-1}$). Photo-autotrophic strains were then tested for the ability/inability to grow on minimal medium (HSM).

Chloroplast biolistic transformation of *Chlamydomonas* was performed with a helium-driven particle gun. 10^8 cells of TAP-grown cy6Nac2.49 were plated on solid agar TAP supplemented with 100 μg/ml spectinomycin (TAP+Spc100) and bombarded with tungsten particles coated with 1 μg pRS1_rcy_aadA plasmid DNA. After 2 weeks in dim light (5 $\mu Em^{-2}s^{-1}$), single colonies were picked and re-cloned four times on TAP+Spec 100 medium, then cultured at 25° C. in dim light (5 $\mu Em^{-2}s^{-1}$) to ensure that the strains were homoplasmic for the selectable marker. To test for photoautotrophic growth, cells were plated on solid HSM medium and grown at 25° C. under medium light (45 $\mu Em^{-2}s^{-1}$). In the case of transformation of Ind41_18, 10^8 TAP-Cu^{+2} cells were plated on solid TAP-Cu^{+2} medium supplemented with 100 μg/ml spectinomycin (TAP-Cu^{+2}+Spc 100) and bombarded with tungsten particles coated with 1 μg pcg12 plasmid DNA. After 2 weeks in dim light (5 $\mu Em^{-2}s^{-1}$), single colonies were picked and re-plated three times on TAP-Cu^{+2}+Spc 100 medium and cultured at 25° C. under dim light (5 $\mu Em^{-2}s^{-1}$).

Growth Analysis

For growth analysis of wt, nac2-26, cy6Nac2.49 strains, cells were grown in TAP-Cu^{+2} medium to a density of 2-4×10^6 cells/ml, then diluted to a density of 1×10^6 cells/ml, followed by 10× serial dilution so that the final dilution was estimated to contain 100 cells when plated. Ten μl aliquots of each dilution were then spotted onto the appropriate solid agar plates, and grown under high light at 25° C. for 10 days. In the case of the Ind41 and Ind41-18 strains, 10^3 cells were plated on the appropriate medium and cultured at 25° C. under continuous illumination (100 $\mu Em^{-2}s^{-1}$) for 10 days. For growth analysis of the inducible aadA transgenic lines, experiments were performed. In brief, wt, Ind41-18 and Ind_aadA transgenic lines were grown in either TAP-Cu^{+2} or TAP liquid medium then transferred 3 times to either fresh TAP-Cu^{+2} or TAP liquid media. Serial dilutions of TAP and TAP-Cu^{+2} grown cultures were plated on TAP medium or TAP-Cu^{+2} solid agar plates supplemented with increasing concentrations of spectinomycin (0-1000 μg/ml) and cultured at 25° C. in under continuous illumination (100 $\mu Em^{-1}s^{-2}$) for 10 days.

Fluorescence Transients

Fluorescence transients were performed. Cells grown on TAP agar in dark were analyzed with a Plant Efficiency Analyzer (PEA, Hansatech Instruments, UK) after dark adaptation for 5 minutes.

RNA Analysis

Isolation of total RNA from wt, nac2-26, cy6Nac2.49, Ind41_18 and Ind_aadA_117 strains was achieved using the RNA Plant Mini RNA extraction kit according to manufacturer's instructions (Qiagen Ghmb, Germany). In the case of

RNA samples taken during time course experiments, 10^8 cells were centrifuged at 3000 g and processed using RNAeasy RNA protection solution according to manufacturer's instructions (Ambion, USA).

RNA blot analysis was performed. RNA (2 μg) was electrophoresed in a 1.2% agarose-4% formaldehyde gel in 1× MOPS buffer, then transferred to a Hybond N+ nylon membrane (Amersham, USA) in 20×SSC buffer and UV-cross-linked to the membrane using a Stratalinker cross-linking oven. Pre-hybridization and hybridization of the membrane was carried out at 65° C. in modified Church's hybridization solution (0.5 M phosphate buffer (pH 7.2), 7% SDS (w/v), 10 mM EDTA). A 380 by DNA fragment of psbD was isolated by digesting plasmid pks-108#14 with AccI and StyI for use as a probe and labeled with [α^{32}P]dATP using the random priming technique. The 685 bps DNA fragment of Cyc6 cDNA was labeled with [α^{32}P]dCTP using the random priming technique. A 804 NcoI/SphI fragment of aadA coding sequence was isolated by digestion of the pcg12 plasmid for use as a probe and labeled with [α^{32}P]dATP using the random priming technique. A 693 bps fragment of atpB was isolated by digesting pcg12 with EcoRV and HpaII for use as a probe and labeled with [α^{32}P]dATP by random priming. After hybridization, membranes were washed at 65° C. for 10 min with high stringency washing buffer [0.1% SDS, 0.1% SSC].

Protein Analysis

Total protein extracts of *Chlamydomonas* strains wt, nac2-26, cy6Nac2.49, Ind41_18, and Ind_aadA_117 were prepared by collecting 3×10^6 cells in a 1.5 ml Eppendorf tube and resuspending the pellet in a 2× solution of Sigma protease inhibitor cocktail (Sigma-aldrech, USA) followed by lysis in an equal volume of cell lysis buffer (100 mM Tris-HCl pH 6.8, 4% SDS) at 37° C. for 30 minutes. To pellet cell debris, the samples were centrifuged at 10,0000 g for 5 min and the supernatant was used as total protein extract. To determine protein concentration 5 μl of supernatant was assayed using Bradford method (Bio-Rad Protein Assay, BioRad, USA).

For immunoblot analysis, 20 μg total protein was separated on a 12% SDS poly-acrylamide gel and transferred to Protran 0.45 μm nitrocellulose membrane (Schleicher and Schuell). When Nac2 antibody was employed, 80 μg protein was loaded on 8% poly-acrylamide gels. Membranes were blocked in Tris-buffered saline solution (containing 5% of non-fat dry milk and 0.1% Tween-20 (TBS-T). For the primary antibody reaction, dilution in TBS-T was as follows: D2 antibody, 1:10,000 dilution; D1, antibody 1:10,000; Nac2 antibody, 1:10,000; PsaA antibody, 1:10,000; AtpB antibody, 1:10,000; RUBISCO-Holo antibody, 1:50,000. Incubation was performed for 1 hr at room temperature. Subsequently, the membrane was washed five times for 5 minutes in TBS-T containing 1% non-fat dry milk. For the secondary antibody reaction, the membrane was incubated for 1 hr at room temperature with peroxidase-linked anti-rabbit IgG (in TBS containing 1% non-fat milk) at a final antibody dilution of 1:10,000. The membrane was washed 5 times for 5 minutes in TBS and the signal was visualized by enhanced chemiluminescence.

Copper-mediated Repression and Time-course Experiments

To follow the copper-mediated repression over time of Cy6Nac2 in the cy6Nac2.49 transgenic strain cy6Nac2.49 cells were grown in TAP-Cu+2 medium to a density of 4×10^6 cells/ml, diluted in fresh TAP-Cu+2 media to a density of 5×10^5 cells/ml, then split into two independent cultures, one of which was left untreated while the other had copper added to the growth medium to a final concentration of 6 μM. Time points were then taken for each culture every 8 hours for 40 hours. Two independent samples were used for F_v/F_M measurements and the average for each culture was determined at the indicated time points.

Experiments to test the copper-mediated induction of cy6Nac2.49 were carried as described for the copper-mediated repression experiments except that pre-cultures of cy6Nac2.49 were grown in TAP medium and the cells were washed two times in copper-depleted medium before dilution in TAP-Cu^{+2} medium at a concentration of 5×10^5 cells/ml. To initiate the time course experiment, cells were split into two separate cultures and copper was added to a final concentration of 6 μM in one of them.

Hydrogen Measurements

The hydrogen production in the cy6Nac2.49 strain was compared with that of the wild type under sulfur deprivation. In the cy6Nac2.49 culture used in FIG. **32**, 20 μmol H_2/L was produced during one cycle corresponding to a maximal rate of 1 mmol H_2 mol^{-1} Ch1 s^{-1}. These rates varied from one experiment to another and reached in some cases 3.1 mmol H_2 mol^{-1} Ch1 s^{-1}. Under conditions permissive for photosynthesis (Cu-deprived medium) the net rate of oxygen evolution was 23 mmol O_2 mol^{-1} Ch1 s^{-1} in Cy6Nac2.49 cells. This rate was 1.5-2 fold higher in wild-type cells. Thus, the maximal rate of hydrogen production ranged between 4 and 13% of the rate of net oxygen production. In the case of a culture subjected to sulfur starvation for 100 hrs, the average value can be estimated at 4 mmol H_2 mol^{-1} Ch1 s^{-1}. If one compares the 20 μmol H_2/L produced by cy6Nac2.49 cells during one cycle with the 4 mmol H_2/L produced during 100 hrs in a sulfur-starved wild-type culture, it is apparent that in order to achieve a similar hydrogen production, the cy6Nac2.49 system still needs further improvement, either a higher efficiency per cycle or a close enchainment of cycles. It is in principle possible to modify genetically *Chlamydomonas* so as to improve hydrogen production e.g. by using state transition mutants blocked in state 1 which are unable to perform cyclic electron flow or by driving one of the Calvin-Benson enzymes with the Cyc6 promoter. In this way carbon assimilation would be diminished at the same time as PSII activity and competition for electrons during the hydrogen production phase would be decreased.

TABLE 2

List of oligonucleotides.

Name	Sequence 5'->3' (SEQ ID NOS: 2-8, respectively, in order of appearance)	Restriction Site
RS1	5'GG***ATCGAT***GCAGGCAGTGGCGGTAC C3'	EcoRI
RS2	5'GATATGTACCGATCGCAATTGTCAT AATTTTATTAATCTTAAAAC3'	N/A
RS3	5'GTTTTAAGATTAATAAAATTATGAC AATTGCGATCGTACATATC3'	N/A
RS4	5'GG***CAGCTG***TTAAGAAGTTACAACCT TC3'	PvuII
Cy6Pro-1		XbaI
Nac2-Cyc6-1	5'CGGTAGAGCCCC***CATATG***GATGGAG TAGGT3'	NdeI

TABLE 2-continued

List of oligonucleotides.

Name	Sequence 5'->3' (SEQ ID NOS: 2-8, respectively, in order of appearance)	Restriction Site
Nac2-Cyc6-2	5'ACCTACTCCATC**CATATG**GGGGCTC TA3'	NdeI
Nac2(+1260)	5'ACCACAGAGCCCTGCCAG 3'	N/A

No natural inducible chloroplast gene expression system is available for *Chlamydomonas*. Such a system was developed by taking advantage of the properties of the nucleus-encoded chloroplast Nac2 protein. This protein is required for processing and stable accumulation of the psbD mRNA which encodes the D2 reaction center polypeptide of PSII. The target site of Nac2 is comprised within the 74 nucleotide psbD 5'UTR. Fusion of this 5'UTR to another coding sequence renders expression of this gene dependent on Nac2. The Nac2 coding sequence has been fused to the Cyc6 promoter of the cytochrome C_6 gene whose expression is induced by copper depletion, anaerobiosis and also by addition of nickel, but which is repressed under copper replete conditions. Because of the specificity of Nac2 for the psbD 5'UTR, this system can be used in principle for the inducible expression of any chloroplast gene by fusing its coding sequence to the psbD 5'UTR.

As described above, the Cyc6-Nac2 construct was inserted into a plasmid containing the aphVIII gene conferring resistance to paromomycin (FIG. **28**A). This plasmid was used for transformation of the *Chlamydomonas* nac2-26 mutant using paromomycin resistance for selection. Amongst 55 transformants tested, two displayed proper control of Nac2 expression by copper. The growth properties of one of these transformants, cy6Nac2.49, of WT and of the nac2-26 mutant are discussed above (FIG. **28**B). As expected, all three strains grow on TAP medium with and without copper and the transformants also grow in the presence of paromomycin because they contain the selectable marker aphVIII. Only WT cells grow on minimal medium containing copper. However, growth of the cy6Nac2.49 strain is restored on minimal medium lacking copper. Growth can also be restored by adding nickel because the Cyc6 promoter is induced by this metal. The level of psbD expression was determined by RNA blot hybridization under different growth conditions (FIG. **28**C). As expected psbD RNA is undetectable in the nac2-26 mutant strain. In contrast in the cy6Nac2.49 strain, expression of psbD follows that of Cyc6 and is induced in the absence of copper or under anaerobic conditions (FIG. **28**C). The level of the psbD product D2 was examined by immunoblotting using D2 antiserum (FIG. **28**D). D2 protein is undetectable in nac2-26 cells grown on TAP plates under all conditions. However in cy6Nac2.49, it accumulates to 20% of wild-type levels when cells are grown in the absence of copper or under anaerobic conditions (FIG. **26**). On minimal medium the induction of D2 is slightly lower. As expected other PSII proteins such as CP47 follow a similar pattern as D2 because it is known that these proteins are unstable in the absence of the D2 protein. In contrast the level of the Rubisco protein (RbcL) is not affected in nac2-26 (FIG. **28**D).

To assess the time required to deplete cells of PSII upon arrest of Nac2 synthesis, cells of cy6Nac2.49 were first grown in copper-depleted TAP medium. Under these conditions PSII is synthesized and accumulates. The culture was split in half and one culture was maintained under copper deprivation whereas copper was added to the other culture. The time course of cell density and the F_V/F_M ratio (variable/maximal fluorescence), which provides an estimate of PSII quantum yield, was determined at various time points (FIG. **29**A). In the presence of copper the F_V/F_M ratio declined to a minimal value within 32 hours. During this period, cells divided 3-4 fold under both conditions and reached stationary phase. Cell extracts were prepared at various times for RNA and protein analysis. The levels of Cyc6 and psbD RNA were significantly decreased 8 hrs after copper addition and were undetectable thereafter (FIG. **29**B). Other chloroplast RNAs (atpB, rRNA) were stable under these conditions. Immunoblotting revealed that the amount of D2 diminished after copper addition with a lag compared to the decrease of its mRNA (FIG. **29**C) and the other PSII core protein D1 also decreased. As expected a decrease in Nac2 was also observed. In contrast chloroplast proteins from PSI (PsaA) and Rubisco were stable (FIG. **29**C).

In a reciprocal experiment, cells grown in the presence of copper were transferred to TAP medium lacking copper and the time course of cell density and of F_V/F_M was determined. F_V/F_M started to increase only after a lag of 25 hours which is presumably due to the time needed to deplete the internal cellular copper reserve (FIG. **30**A). RNA and protein from cell extracts at different time points were examined by RNA blot analysis and protein immunoblotting (FIG. **30**B, C). While the Cyc6 RNA was detectable after 16 hours, there was a delay in psbD RNA accumulation and PSII activity presumably due to the fact that a threshold level of Nac2 is required for the accumulation of psbD mRNA and D2 synthesis.

Inducible Expression of Chloroplast Genes Unrelated to PSII

While the Nac2 system can be used to deplete PSII in a reversible manner, we tested whether it can be extended to any other chloroplast gene. This is in principle possible because the Nac2 protein acts specifically on the psbD 5'UTR and can drive chimeric psbD 5'UTR reporter genes. Hence it should suffice to fuse the psbD promoter and 5'UTR to the gene of interest. However, under those conditions PSII no longer accumulates because of the nac2-26 mutation which leads to the loss of psbD RNA. To circumvent this situation, the petA promoter and 5' UTR were fused to the psbD coding sequence and this construct was introduced into a modified version of the p108-14 chloroplast transformation vector. In this vector the recyclable aadA cassette is inserted upstream of the psbD gene which is driven by the petA promoter and 5'UTR (FIG. **27**A). This DNA was inserted into the chloroplast genome by biolistic transformation using the aadA cassette as selectable marker. In this way the endogenous psbD gene was replaced by the petA-psbD construct and thus accumulation of its transcript was no longer dependent on Nac2. Transformants were restreaked three times on spectinomycin plates and the homoplasmicity was tested by DNA blot and PCR analysis and one of the transformants, Ind41 was selected. The aadA cassette used was flanked by two repeats. To allow for the excision of the cassette, the homoplasmic transformant Ind41 was plated repeatedly on medium lacking spectinomycin. In this way a strain was obtained, Ind41_18, which is sensitive to spectinomycin because it lacks the aadA cassette. The growth properties of Ind41, Ind41_18 and cy6Nac2.49 were tested on different media (FIG. **27**B). As expected Ind41 grows in the presence of spectinomycin in contrast to Ind41_18 which is sensitive to the antibiotic. Moreover both Ind41 and Ind41_18 grow on HSM minimal medium with or without copper. RNA blot analysis revealed that the chimeric petA-psbD RNA in Ind41_18 accumulates under all conditions independent of the Cyc6 RNA level

(FIG. **27**C). The psbD RNA is larger because the size of the petA 5'UTR exceeds that of the psbD 5'UTR. Immunoblot analysis revealed that D2 and D1 proteins accumulate to the same level under all conditions tested, in particular when Nac2 is not expressed (FIG. **27**D).

Next, the aadA cassette fused to the psbD promoter and 5'UTR was introduced into this strain by transformation using the cg12 vector (FIG. **31**A). Growth of the transformants Ind_aadA-117 and of Ind41__18 was tested on TAP plates containing increasing amounts of spectinomycin with and without copper (FIG. **31**B). All strains grow in the absence (FIG. **31**B) or presence of copper. As expected the Ind_aadA-117 grows on spectinomycin plates at concentrations of 250 μg/ml or higher only in the absence of copper. A faint growth was also observed in the presence of copper on plates containing 100 μg/ml spectinomycin. RNA blot analysis revealed that aadA RNA accumulates only under inducing conditions for the Cyc6 promoter (FIG. **31**C). Protein levels of D2, D1, CP47, and RbcL were largely unaffected but Nac2 was only detected under inducing conditions (FIG. **31**D). Because of a lack of reliable aadA antibody, the amount of this protein was assayed by measurements of aminoglycoside adenyl transferase activity. The activity was significantly elevated under conditions when Nac 2 is expressed (Table 3).

TABLE 3

Aminoglycoside adenyl transferase activity in Ind41_aadA-117 under inducing and repressing conditions.

Strain	+Cu	−Cu
WT-aadA	207.0 +/− 49.5 (3)	192.6 +/− 51.4 (4)
Ind41_18	9.2 +/− 4.1 (4)	12.2 +/− 7.9 (4)
Ind41_117	24.2 +/− 12.5 (4)	274.3 +/− 90.6 (7)

Extracts from WT-aadA, Ind41__18 and Ind41_aadA-117strains were assayed for aadA activity and for total protein content as described above. The activity is indicated as cpm incorporated per μg protein. Numbers of independent measurements are indicated in parenthesis.

The Inducible Chloroplast Gene expression System can be Used to Trigger Hydrogen Production

Chlamydomonas is able to induce hydrogenase and produce hydrogen under anaerobic conditions in the light. It was therefore tested whether the inducible Nac2 system could be used to turn off PSII activity and O_2 evolution, so that respiration would lead to anaerobic conditions suitable for induction of hydrogen production. Cells of cy6Nac2.49 were grown in TAP medium lacking copper to a concentration of 2×10^6 cells/ml. Copper was added and the cells were transferred into a chamber connected to a mass spectrometer and illuminated with white light (250 μE $m^{-2}s^{-1}$). In this system the bottom of the chamber is sealed with a polypropylene membrane which allows dissolved gases to diffuse directly into the ion source of the mass spectrometer. In this way the abundance of O_2 and H_2 could be measured at discrete time intervals. Because the chamber was closed and because copper repressed the synthesis of PSII, O_2 evolution diminished. Within a period of 200 min the O_2 was consumed by respiration. An anaerobic state was reached which led to the synthesis of active hydrogenase and H_2 production (FIG. **32**A). The maximal rate of hydrogen production ranged between 1 and 3.1 mmol H_2 mol^{-1} Ch1 sec^{-1}, slightly lower than that obtained with sulfur-starved cells, and of much shorter duration (around 1.5 h vs 3-4 days).

An interesting feature of the Cyc6 promoter is that it is also induced under anaerobic conditions even in the presence of copper. It would therefore be expected that once anaerobic conditions have been reached and hydrogenase is induced in cy6Nac2.49 cells, Nac2 synthesis resumes, PSII is synthesized and oxygen levels rise thus inactivating hydrogenase and blocking hydrogen production. This was observed. PSII synthesis was switched on during the anaerobic hydrogen production phase with concomitant O_2 evolution and inactivation of hydrogenase so that the hydrogen levels remained constant (FIG. **32**A). As a control, the same cell culture was examined without addition of copper. Under these conditions no hydrogen was produced (FIG. **32**B) with a constant production of O_2 and a gradual decrease of CO_2 as observed in the copper-treated cells. Because the Cyc6 promoter is expected to be switched off under aerobic conditions in copper-replete medium, a new cycle of hydrogen production would be expected. To test this possibility further cy6Nac2.49 cells were grown in TAP medium lacking copper in a sealed vessel for 50 hrs and measurement of hydrogen and oxygen were performed by mass spectrometry. The results suggest that two successive phases of hydrogen and oxygen production occurred.

Although the invention has been described in detail with reference to certain preferred embodiments, variations and modifications exist within the scope and spirit of the invention as described and defined in the following claims.

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What is claimed is:

1. A method for expressing a heterologous protein in the plastid of a cell, the method comprising the steps of

providing a *Chlamydomonas* cell that comprises in its plastid a construct comprising a promoter and a psbD 5'UTR operably linked to a nucleic acid encoding a heterologous protein;

introducing into the nucleus of the cell a recombinant nucleic acid comprising an inducible promoter operatively linked to a nucleic acid encoding Nac2;

contacting the cell with an inducer or treating the cell under conditions that result in the removal of a repressor;

wherein the inducer or the repressor associates with the inducible promoter in the nucleus;

wherein association of Nac2 with the mRNA encoding the heterologous protein enhances expression of the mRNA, resulting in the production of the heterologous protein in the plastid.

2. The method of claim **1**, wherein the cell has an inoperative or missing copy of the endogenous nucleic acid encoding Nac2.

3. The method of claim **1**, wherein the plastid is selected from the group consisting of a chloroplast, a leucoplast, an amyloplast, an etioplast, an elaioplast, and a chromoplast.

4. The method of claim **1**, wherein the inducible promoter has the sequence of SEQ ID NO:11.

5. The method of claim **1**, wherein the inducer is a reduction in the concentration of oxygen to a predetermined level.

6. The method of claim **1**, wherein the inducer is applied and removed for a plurality of cycles, wherein a cycle comprises applying and removing the inducer.

7. The method of claim **1**, wherein the heterologous protein is selected from the group consisting of a pharmaceutical agent, an industrial enzyme, an enzyme involved in chloroplast maturation or degradation, a nutraceutical, a defense product for the cell, an enzyme involved in amino acid synthesis, a protease, an antibody, a vaccine, and an antimicrobial agent.

8. A system for expressing a heterologous protein in the plastid of a recombinant *Chlamydomonas* cell, the system comprising

an exogenously added or removed inducer for an inducible promoter; and

a *Chlamydomonas* cell that comprises in its plastid a construct comprising a promoter and a psbD 5'UTR operably linked to a nucleic acid encoding a heterologous protein and comprises in its nucleus a recombinant nucleic acid comprising the inducible promoter operatively linked to a nucleic acid encoding Nac2.

9. The system of claim **8**, wherein the cell has an inoperative or missing copy of the endogenous nucleic acid encoding Nac2.

10. The system of claim **8**, wherein the plastid is selected from the group consisting of a chloroplast, a leucoplast, an amyloplast, an etioplast, an elaioplast, and a chromoplast.

11. The system of claim **8**, wherein the inducible promoter has the sequence of SEQ ID NO:11.

12. The system of claim **8**, wherein the inducer is a reduction in the concentration of oxygen to a predetermined level.

13. The system of claim **8**, wherein the heterologous protein is a protein involved in the production of hydrogen gas.

14. The system of claim **8**, wherein the heterologous protein is selected from the group consisting of a pharmaceutical agent, an industrial enzyme, an enzyme involved in chloroplast maturation or degradation, a nutraceutical, a defense product for the cell, an enzyme involved in amino acid synthesis, a protease, an antibody, a vaccine, and an antimicrobial agent.

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